HHS Public Access

Author manuscript

Laryngoscope. Author manuscript; available in PMC 2024 January 01.

Published in final edited form as:

Laryngoscope. 2023 January ; 133(Suppl 1): S1–S11. doi:10.1002/lary.30242.

Oral and inhaled fosamprenavir reverses pepsin-induced damaged in a laryngopharyngeal reflux mouse model

Nikki Johnston, PhD[1,2], **Tina L. Samuels, MS**[1], **Christopher J. Goetz, BS**[3], **Leggy A. Arnold, PhD**[4], **Brian C. Smith, PhD**[3], **Donna Seabloom, BS**[5], **Beverly Wuertz, BS**[5], **Frank Ondrey, MD, PhD**[5], **Timothy S. Wiedmann, PhD**[6], **Nemanja Vuksanovic, PhD**[4], **Nicholas R. Silvaggi, PhD**[4], **Alexander C. MacKinnon, MD, PhD**[7], **James Miller, MD, MPH**[8], **Jonathan Bock, MD**[1], **Joel H. Blumin, MD**[1]

[1]Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, Milwaukee, WI,

[2]Department of Microbiology and Immunology, Medical College of Wisconsin,

[3]Department of Biochemistry, Medical College of Wisconsin,

[4]Department of Chemistry and Biochemistry, Milwaukee Institute for Drug Discovery, University of Wisconsin, Milwaukee, WI,

[5]Department of Otolaryngology Head and Neck Surgery, University of Minnesota, Minneapolis, MN,

[6]Department of Pharmaceutics, University of Minnesota,

[7]Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL,

[8]Department of Pathology, Medical College of Wisconsin

Abstract

Objective: More than 20% of the US population suffers from laryngopharyngeal reflux. While dietary/lifestyle modifications and alginates provide benefit to some, there is no gold standard medical therapy. Increasing evidence suggests that pepsin is partly, if not wholly, responsible for damage and inflammation caused by laryngopharyngeal reflux. A treatment specifically targeting pepsin would be amenable to local, inhaled delivery and could prove effective for endoscopic signs and symptoms associated with nonacid reflux. The aim herein was to identify small molecule inhibitors of pepsin and test their efficacy to prevent pepsin-mediated laryngeal damage *in vivo*.

Methods: Drug and pepsin binding and inhibition were screened by high-throughput assays and crystallography. A mouse model of laryngopharyngeal reflux (mechanical laryngeal injury once weekly for 2 weeks and pH7 solvent/pepsin instillation 3days/week for 4 weeks) was

Corresponding author: Nikki Johnston, Ph.D., njohnston@mcw.edu, 414-955-4075 (phone), 414-955-6535 (fax), Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226.

Competing Interests: N.J. is an inventor on an International Patent Application: PCT/US2021/027758, Aerosolized formulations of HIV protease inhibitors for the treatment of airway reflux, filed April 16, 2021. Since completion of this study, N.J. became co-founder, Chief Scientific Officer and an investor in N-Zyme Biomedical, and T.S. an investor in N-Zyme Biomedical. The other authors have no financial relationships or conflicts of interest to disclose.

provided inhibitor by gavage or aerosol (fosamprenavir or darunavir; 5days/week for 4 weeks; n=3). Larynges were collected for histopathologic analysis.

Results: HIV protease inhibitors amprenavir, ritonavir, saquinavir and darunavir bound and inhibited pepsin with IC_{50} in the low micromolar range. Gavage and aerosol fosamprenavir prevented pepsin-mediated laryngeal damage (i.e. reactive epithelia, increased intraepithelial inflammatory cells, and cell apoptosis). Darunavir gavage elicited mild reactivity and no discernable protection; aerosol protected against apoptosis.

Conclusions: Fosamprenavir and darunavir, FDA-approved therapies for HIV/AIDS, bind and inhibit pepsin, abrogating pepsin-mediated laryngeal damage in a laryngopharyngeal reflux mouse model. These drugs target a foreign virus, making them ideal to repurpose. Reformulation for local inhaled delivery could further improve outcomes and limit side effects.

Keywords

Laryngopharyngeal reflux; LPR; pepsin; fosamprenavir; Lexiva™; darunavir; Prezista™

INTRODUCTION

Laryngopharyngeal reflux (LPR), the backflow of gastric contents into the laryngopharynx, is an important health problem. LPR affects both children and adults, and the clinical spectrum is extensive. Unlike patients with gastroesophageal reflux (GER) which is limited to the esophagus, many LPR patients do not experience acid indigestion but present with symptoms due to chronic laryngeal irritation, such as chronic cough, throat-clearing, post-nasal drip, dysphonia, globus, dysphagia, and dyspnea[1–3]. Significant evidence supports the contribution of chronic LPR to serious and life-threatening illness including airway stenosis, reactive airway disease, and laryngeal cancer[4–14]. LPR is estimated to affect more than 20% of the United States population and contribute to 10% visits to otolaryngologists[15–17]. The economic burden of LPR is over $52 billion per year, which is 5.6-fold greater than that of GER; 52% of the burden is attributed to proton pump inhibitors (PPIs)[18,19].

While PPI therapy is a mainstay in the treatment of GER disease (GERD), its efficacy for LPR is poor[20–23]. In clinical practice, it was believed that patients with reflux laryngitis require higher doses and longer trials of PPIs than those with typical GERD given the assumption that the upper airway is more sensitive to acid reflux than the esophagus[3,24,25]. However, placebo-controlled trials have failed to demonstrate therapeutic benefit of PPIs[26–31]. While Reichel et al. and Lam et al. reported symptom improvement in randomized, double-blind, placebo-controlled trials[32,33], Vaezi argued that improvement was for heartburn rather than throat symptoms[34]. Where laryngeal symptom improvement has been reported it was found proportionally higher in GERD patients than in those without GERD[35,36]. Given the paucity of data supporting acid-suppression therapy for extraesophageal symptoms, the American Gastroenterological Association guidelines for GERD recommend against its use for acute treatment of patients with potential extraesophageal reflux (EER) syndromes (laryngitis, chronic cough) absent typical GERD symptoms[37]. Despite such advice, treatment for LPR frequently continues to involve empiric therapy with PPIs[38,39].

While the acidity of reflux alone can damage the upper airways, combined multichannel intraluminal impedance-pH (MII-pH) monitoring has demonstrated that many episodes of LPR are nonacidic, and that weakly and nonacidic reflux is associated with persistent symptoms in acid-suppressed patients.[40–43] These symptoms are alleviated by anti-reflux surgery[44–50] and may be ameliorated by less invasive strategies that limit reflux occurrence or neutralize reflux constituents beyond acid (e.g. dietary and lifestyle modification and over-the-counter alginate products).[51–54] Thus, one or more nonacid components of gastric refluxate must have a role in laryngeal damage. There is increasing evidence that pepsin, which is present in all refluxate[55], is partly, if not wholly, responsible for damage and inflammation caused by LPR[20,39,56–60].

Pepsin is a proteolytic enzyme which is synthesized and secreted as the zymogen pepsinogen by chief cells in the gastric fundus and subsequently cleaved upon introduction to the acidic stomach lumen to produce pepsin. Pepsin is maximally active at pH2 and retains activity up to pH6.5. While stable at pH8, pepsin is irreversibly inactivated at higher pH[58,61]. The stomach and esophagus have intrinsic defenses against pepsin (mucus, peristalsis, and bicarbonate secretion), however laryngeal tissues do not[62]. Pepsin is thought to play a key role in mucosal damage and inflammation during nonacidic reflux[8,9,58,59,62–74]. At neutral pH, pepsin is taken up by laryngeal and hypopharyngeal cells by receptor-mediated endocytosis and retained in intracellular vesicles of low pH where it is presumed to be reactivated[58,67,68]. The consequence is chronic inflammation, which in turn, gives rise to symptoms. Endocytosed nonacidic pepsin induces a proinflammatory cytokine gene expression profile in hypopharyngeal cells similar to that which contributes to disease severity during GERD[59]. Inhibition of the proteolytic activity of pepsin abrogates this damage and inflammation[8,60,68,75–78]. The on-surgical treatment options for nonacid reflux

With compelling evidence of nonacid proximal reflux of pepsin and its association with laryngeal and pharyngeal symptoms and endoscopic findings, the significant cost and risk of prolonged PPI therapy which continues to date despite its inefficacy in the absence of a gold standard medical therapy, and the limitations of alternative non-surgical treatment options such as the short-lived activity of over-the-counter products intended to provide temporary relief and the burden of adherence to dietary and lifestyle modifications, a new medical treatment which specifically targets pepsin would be of great value[18,20,39,46,53,59,65,67,68]. This new approach would be amenable to local treatment of readily accessible airways affected by LPR allowing lower dosing, the advantage of which is self-evident in that targeted delivery would simultaneously increase efficacy and limit systemic side effects.

We and others have discussed the promise of inhibitors of peptic activity and/or receptor antagonists as potential new therapeutics for LPR[20,65,68,79]. Given evidence that airway damage during EER is more closely associated with pepsin than acid, we hypothesize that a drug that targets pepsin will be effective for signs and symptoms associated with nonacid reflux. Local inhaled administration of such a drug would be more efficacious than oral. Herein, therapeutic compounds were screened for pepsin binding and inhibition. Specific HIV protease inhibitors that inhibited pepsin were administered orally and by inhalation in an LPR mouse model to assess their potential for the treatment of LPR.

METHODS

Binding and activity assays

To examine whether HIV protease inhibitors bound and inhibited pepsin, we developed assays based on fluorescence polarization which measures size-dependent molecular rotation thereby permitting detection of degradation, association and dissociation events[80]. A competitive binding assay was designed employing pepstatin, an inhibitor of sub-nanomolar affinity[81]. Pepstatin-Alexa647 was synthesized by dissolving 1mg pepstatin A (Sigma-Aldrich) in a 50:50 mixture of dimethylformamide (DMF) and dimethylsulfoxide (DMS) followed by the addition of N,N,N′,N′-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (0.6mg) and trimethylamine (10μL) DMF. The mixture was stirred for 1 hour, after which 1mg Alexa Fluor 647 Cadaverine, Disodium Salt (ThermoFisher Scientific) was added. After 2 hours, the solvents were evaporated under high vacuum (35°C) and residue partially dissolved in 10% methanol and transferred onto a C18 cartridge (Waters Corporation, Milford, MA). Increasing percentages of methanol were used for the elution. Pepstatin-Alexa647 eluted at 45% methanol. An enzymatic inhibition assay was designed using casein substrate.[82] Bovine alpha casein (Sigma-Aldrich, St. Louis, MO) was labeled with Alexa Fluor 647 Carboxylic Acid, Succinimidyl Ester (ThermoFisher Scientific, Waltham, MA) as described.[82] Briefly, the two were combined at 2.5ug/mg label to protein ratio in 0.1M sodium bicarbonate for 15 minutes and labeled casein was separated from unbound label in a Sephadex G-25 (Sigma-Aldrich) column comprised of 90 × 5mm packed beads in a glass Pasteur pipette, eluted with dPBS pH7.4 (ThermoFisher Scientific). The fast-moving band (casein-bound fluorophore) was collected in ~0.4 ml volume. Concentration of resultant probe (casein-Alexa647 in PBS-azide) was estimated via spectrophotometry using Beer's law (Implen Nanophotometer, Implen, Inc. Westlake Village, CA).

Assays were optimized using ranges of 0.3–1000μM unlabeled pepstatin, 100–500nM pepstatin-Alexa647 or casein-Alexa647 probe, 0.003–3U/μl porcine pepsin (Worthington Biochemical Corporation, Lakewood, NJ), and 5–37.5% DMSO (HIV protease inhibitor diluent) in 0.1 M HCl, pH 1 with 0.01% v/v Tween-20 in 20 μl volumes in 384-well black optical plates (Nunc, Roskilde, DK) and read on a BioTek Cytation 5 (BioTek Instruments, Winooski, VT) with far red FP filter cube (excitation/emission 620/680nm). Unlabeled pepstatin dose-response curves were used to ensure that the assays were responsive to pepsin inhibition. Conditions yielding maximal dynamic assay range were used to assess HIV protease inhibitors: 100nM probe, 0.03U/ul porcine pepsin A, 37.5% DMSO for competitive binding assay, and 200nM probe, 0.01U/μL pepsin, 5% DMSO for peptic activity assay. The HIV protease inhibitors (amprenavir, ritonavir, lopinavir, saquinavir mesylate, nelfinavir mesylate hydrate, darunavir ethanolate, indinavir sulfate salt hydrate; all Sigma-Aldrich) were dissolved in DMSO and tested under optimized assay conditions over three logs concentration. Assays were performed twice with triplicate reactions read for five minutes and mean mP plotted against probe concentration (binding assay) or read at <2minutes intervals over 30minutes with mean mP of plotted over time (activity assay). Half maximal inhibitory concentration (IC_{50}) of inhibitors were calculated from kinetic traces analyzed

using an online tool (https://icekat.herokuapp.com/icekat)[83]. mP was normalized to blank (absent inhibitor) to derive percent bound or activity.

Crystallization

Saturated solutions of HIV protease inhibitors (amprenavir, ritonavir and darunavir ethanolate) were prepared in DMSO and centrifuged for 10 minutes at 31,000 rcf. Supernatants were added to pepsin (200mg/ml in water) at 1.6% (v/v) f.c. Due to poor solubility, a solvent for saquinavir mesylate was selected from the CryoSol screen (Molecular Dimensions, Holland, OH). CryoSol mixture SM2 (consisting of 37.5% v/v dioxane, 25% v/v DMSO, 12.5% v/v ethylene glycol, 12.5% v/v 1,2-propanediol, and 12.5% v/v glycerol) was selected as it provided both high solubility and protein compatible conditions for the co-crystallization mixture. Supernatant of saturated saquinivar solution in SM2 was combined with pepsin at 5% f.c. (v/v). Crystallization conditions were optimized by screening 200mg/ml pepsin in the Salt RX screen (Hampton Research, Viejo, CA). Small bipyramid-shaped crystals formed in 3.5M ammonium chloride and 0.1M sodium acetate trihydrate pH 4.6 after one week at room temperature served as microseed stock for co-crystallization with amprenavir, ritonavir and darunavir ethanolate per previously described methods[84]. Diffraction quality crystals (triangular bi-pyramids, approximately 200 × 100 × 100μm) formed after 2–7days from hanging drops of 2ul pepsin (180–210mg/ml) and 1ul microseed solution serially diluted 10–100x above 3–4M ammonium chloride and 0.1M sodium acetate trihydrate pH4.6. Crystals were cryoprotected by 30% glucose, 5M ammonium chloride and 0.1M sodium acetate trihydrate pH4.6 and plunged in liquid nitrogen. Co-crystallization with saquinavir was performed in 0.1M acetic acid rather than sodium acetate trihydrate as this permitted large crystal formation without a microseed; crystals were cryoprotected by 30% w/v glucose, 5M ammonium chloride and 0.1M sodium acetate trihydrate pH 4.6 and plunged in liquid nitrogen.

Diffraction datasets were collected at Life Sciences Collaborative Access Team (LS-CAT) beamlines at the Advanced Photon Source (APS), Argonne National Laboratory, equipped with MAR 300 CCD or Dectris Eiger 9M detectors and data were indexed, integrated and scaled using MOSFLM[85] or HKL2000[86].

Specifically, for pepsin:amprenavir, a 1.9Å diffraction data set was collected at LS-CAT beamline 21-ID-F with a MAR 300 CCD detector using a 50 × 50μm beam at a wavelength of 0.97872Å. A total of 262 frames were collected from $\varphi = 0$ to 130.5^{o} with an oscillation range of 0.5° and detector distance of 250mm. Exposure time was 0.5 seconds. Diffraction data were indexed, integrated and scaled using MOSFLM.

For pepsin:ritonavir, a 2.1Å diffraction data set was collected at LS-CAT beamline 21-ID-D with Dectris Eiger 9M detector using a 50 × 50 μm beam at 1.12721Å. 900 frames were collected from $\varphi = 0$ to 180^{o}, while oscillating at a rate of $1\ ^{o}$/sec and slicing of 5 images/^{o}. Crystal-to-detector distance was 160mm. Diffraction data were indexed, integrated and scaled using MOSFLM.

For pepsin:darunavir, a 1.9Å diffraction data set was collected at LS-CAT beamline 21-ID-G with MAR 300 CCD detector and 50 × 50μm beam at 0.97856Å. 900 frames were collected

from φ = 0 to 180^{O} with an oscillation range of 0.2^{O} and detector distance of 260 mm. Exposure time was 0.3 seconds. Diffraction data were indexed, integrated and scaled using HKL2000.

For pepsin:saquinavir, a 1.9 Å diffraction data set was collected at LS-CAT beamline 21-ID-F with MAR 300 CCD detector using a 50 × 50 μm beam at 0.97872Å. 400 frames were collected from φ = 20 to 100^{O} with an oscillation range of 0.2^{O} and detector distance of 200mm. Exposure time was 0.5 seconds. Diffraction data were indexed, integrated and scaled using MOSFLM.

Initial phases were obtained by molecular replacement in PHASER[87]. Unliganded porcine pepsin (PDB ID 4PEP) with B factors reset to 20.00 Å and solvent molecules removed was the search model. Model refinement was performed using phenix.refine (PHENIX[87–89]) and COOT[90,91]. Geometric restraints for compounds were obtained from CCP4 monomer library[92]. Models were validated using MolProbity[93] as implemented in the PHENIX suite. Models of ritonavir and saquinavir were additionally optimized using PDB-REDO server[94] prior to deposition. Electron density maps were generated via POVSCRIPT and POV-Ray and schematic representation by MarvinSketch,(http://www.ChemAxon.com) and Adobe Illustrator CC 2020.

In vivo mouse model

Experiments were approved by the University of Minnesota (UMN) Institutional Animal Care and Use Committee (1712-35415A) and performed at UMN. Three replicate animals per treatment condition were anticipated to suffice for verification of reproducibility in each experiment without excessive use of animal life. The three mice were randomly allocated to treatment groups. No data were excluded from analysis.

Six-week-old female Jackson A/J mice (Jackson Laboratory, Bar Harbor, ME) were fed D-62 powdered Wattenberg diet, 2 g/mouse/day[95] and allowed to acclimate for one week upon arrival prior to experiments. In accord with previously established methods for modeling aerodigestive tract damage attributed to GERD and LPR,[1,95–99] mechanical injury applied during the first two weeks of a four-week treatment course was used to predispose the laryngeal mucosa to chemical injury by pepsin/acid applied throughout the four weeks. When performed in this manner, mechanical injury increases mucosal susceptibility to subsequent chemical injury while leaving little detectable injury at the conclusion of a four-week treatment course.[95] Mechanical injury was performed on all animals (including control) once weekly during the first two weeks of treatment as described (see experimental schema, Figure 1)[95]. Briefly, anesthetized mice were suspended by upper teeth on a slanted board under an operating microscope. Subglottis, glottis, and supraglottis were wounded under 6x magnification using a blunt, bent (135°) needle pulled distally to proximally making a mild abrasion.

In a preliminary experiment to validate the LPR mouse model (i.e. laryngeal damage by pepsin at neutral and acidic pH), 20μl saline (solvent control) or 0.3mg/ml pepsin at pH7.0 or 4.0 were provided to mice (n=3) by laryngeal instillation at 24, 48, and 72 hours after mechanical injury during weeks 1 and 2 (Figure 1); laryngeal instillation without

wounding (3 days/week) continued during weeks 3 and 4. Mice were anesthetized with 225–240mg/kg intraperitoneal Avertin (2,2,2-Tribromoethanol) prior to each wounding and laryngeal instillation. Mice were sacrificed at conclusion of the fourth week.

To test the protective effect of HIV protease inhibitors on pepsin-mediated damage *in vivo*, inhibitors were delivered by aerosol or gavage concurrently with wounding (days 2, 8) and solvent/pepsin instillation (days 3–5, 9–11, 16–18 and 23–25). Aerosol or gavage was provided on days 1–5, 8–12, 15–19, and 22–25, and mice sacrificed day 26. Mice were anesthetized with isoflurane (3% in 2.5LPM, 3–5 minutes prior to procedures) as opposed to Avertin due to frequency. Lexiva and Prezista (hereafter referred to by generic: fosamprenavir and darunavir, respectively) were used for gavage, and respective pure drugs for aerosol (fosamprenavir from Anant Pharmaceuticals, Ambernath, Maharashtra India and darunavir from Ambeed, Arlington Heights, IL). Gavage dose was equivalent to that prescribed to HIV patients (20mg/kg/day fosamprenavir; 8.6mg/kg/day darunavir). Aerosol was generated as described[100]. Briefly, a 10ml suspension of drug in ethanol was placed in the baffle, such that the concentration would remain constant at the equilibrium solubility. Droplets of ethanol containing dissolved drug were generated by an ultrasonic atomizer (nominal frequency 1.7 MHz) and entrained by air at a flow rate of 0.5 LPM with a custom-built glass baffle (UMN Department of Chemistry Glass Shop). The aerosol cloud was then passed through a cylindrical drying column containing an annular ring of charcoal. The ethanol was removed and the emanating dry aerosol particles of pure drug were then directed into the exposure chamber. The mass deposited on filters was measured gravimetrically and total output rate (mg/min) was determined. The aerosol concentration (mass/volume of air) was calculated by dividing the total output rate by the air flow rate (0.5 LPM). The inhaled mass of drug (M_{inh}) for each mouse was defined as M_{inh}=[Aerosol]*RMV*t, where Aerosol is the aerosol concentration of drug, RMV is the respiratory minute volume of the mice (0.025 L/min), and t is the aerosol exposure time. Aerosol concentration was 0.09mg/L fosamprenavir or 1.2mg/L darunavir, therefore given the respiratory minute volume of mice (0.025 L/min), the inhaled mass was 0.93mg/kg/day fosamprenavir or 12mg/kg/day darunavir. Actual mass deposited was not determined but anticipated to be 10% of inhaled mass (the deposition fraction of 1μm aerosol particles in mice).

Tissues were collected, fixed in paraformaldehyde, embedded in paraffin and 4um sections stained with hematoxylin and eosin (H&E) via automated stainer. Slides were reviewed by a board-certified pathologist (JM) blinded to treatment groups.

RESULTS

Binding and Activity Assays

Four of the seven assayed HIV protease inhibitors bound and inhibited pepsin at low micromolar concentrations (Figure 2): amprenavir, darunavir, ritonavir, and saquinavir. The *in vitro* activity of these four HIV protease inhibitors against pepsin provided the foundational support for further study.

Structural data

To aid interpretation of the *in vitro* binding and inhibition data, commercially available porcine pepsin (EC 3.4.23.1) was used for co-crystallization experiments to obtain structural data. Crystallization of human pepsin collected from volunteers failed presumably due to sample heterogeneity. Porcine pepsin shares 86% sequence identity with the human enzyme (PDB ID 1PSN)[101] and its structure is nearly identical (root mean square deviation (RMSD) for all Cα atoms=0.50Å). Minor differences in tertiary structure are localized to a loop of residues (277–282) which is not part of the binding cleft. Residues lining the active site cleft are highly conserved: of 17 making direct contact with inhibitors herein, just two differed (T12 and V291). Thus, porcine was deemed an acceptable substitute for human pepsin for assessing structural biology.

Porcine pepsin was co-crystalized amprenavir, darunavir, ritonavir, and saquinavir (Table 1 and Figure 3). All are peptidomimetics; the alcohol of the central phenylalaninol residue, which mimics the tetrahedral intermediate of peptide bond cleavage, is bound between catalytic aspartate residues, D32 and D215. Binding directionality of each (amino group of phenylalaninol on the prime side of the binding site) was the same as that for pepstatin[101]. Binding relied on van der Waals contacts between side chains of inhibitors and residues lining the binding site; few (5–6) hydrogen bonds were observed. For example, in the pepsin·ritonavir complex (Figure 3), the β-homophenylalanine side chain is bound in the P1 subsite, making van der Waals contacts with F111, F117, and I120. The phenylalaninol side chain is bound in the P1 subsite, contacting I213, M289, V291, and I300. The thiazole and isopropyl-thiazole groups of ritonavir do not have any stabilizing interactions with the active site. The electron density for these groups is correspondingly poorly defined, and the B-factors, which reflect the precision of the atomic positions, for these parts of the molecule are extremely high. The structure of the pepsin·saquinavir complex is similar in that the side chain of the phenylalaninol residue is interacting with the P1’ subsite, but the two ends of the molecule, the quinoline and decahydroisoquinoline moieties, also have poor density and high B-factors. The amprenavir and darunavir structures follow the same pattern. The phenylalaninol residues of both inhibitors occupy the P1’ site, interacting with I213, M289, V291, and I300. The isobutyl groups, mimicking leucine residues, occupy the P1 site, interacting with F111, F117, and I120. In both amprenavir and darunavir, one of the oxygen atoms of the sulfonamide moiety makes a hydrogen bond with the backbone amide of T77. The aniline groups make no polar contacts with the active site. At the opposite end of the molecules where the two compounds differ, the tetrahydrofuran group of amprenavir forms a hydrogen bond with the phenolic oxygen of Y189. The bis-tetrahydrofuran group of darunavir, however, cannot have this interaction with the active site and is limited to van der Waals contacts with I73, T74, I128, and Y189. The structures and binding poses of amprenavir and darunavir were similar and provided no explanation for their disparity in IC_{50}.

In vivo mouse model

Pepsin-mediated laryngeal epithelial damage was observed at pH 4 and 7 in the mouse *in vivo* model which employed pepsin with or without acid exposure following mechanical injury of the larynx (Figure 4). Animals in the pH7 control group had normal laryngeal

epithelium of 1–2 cells thick with cilia present and no inflammation, keratinization, or necrosis; findings indicated no detectable mucosal damage in the control group due to mechanical injury during the first two weeks of treatment or pH7 solvent. Laryngeal epithelium in the pH4 group was reactive, thickened (3–4 cells thick), and keratinized with loss of cilia. That from the pepsin-pH7 group had an intermediate thickness (2–3 cells), evidence of keratinization, increased nuclear to cytoplasmic ratio and loss of polarization. That from the pepsin-pH4 group exhibited total loss of epithelium due to necrosis and inflammatory cell infiltrate.

Fosamprenavir gavage equivalent to the dose used to treat HIV in humans prevented pepsin-mediated laryngeal damage, defined as reactive epithelia, increased intraepithelial inflammatory cells, and apoptosis (Figure 5). Mild reactivity elicited by oral darunavir (absent in darunavir aerosol group; Figure 4) obscured the ability to detect its effect on pepsin-mediated damage. Fosamprenavir aerosol prevented pepsin-mediated laryngeal injury (Figure 4). Darunavir aerosol provided moderate protection against pepsin-mediated damage: while epithelial injury was present (mildly increased intraepithelial inflammatory cells and reactive epithelial cells), no apoptosis was observed as it was in mice treated with pepsin-pH7 and sham inhalation.

DISCUSSION

For the past two decades, the treatment of LPR has focused on suppressing gastric acid production. With the introduction of MII-pH technology, it is now understood that LPR is commonly nonacidic and that nonacid proximal events are associated with laryngeal endoscopic signs and symptoms[39–46,48–50,102]. These findings sparked investigations into the nonacidic components of gastric refluxate.

Although bile induces mucosal damage at weakly and non- acid pH experimentally, it has been argued that “there is no evidence that the same mechanism occurs in the human larynx”[57]. The clinical relevance of experimental findings has been called into question. Unconjugated bile acids, which cause damage at neutral-high pH such as that of the laryngopharynx, are rarely found in gastric refluxate.[56,69] Further, concentrations of bile salts/acids found to damage the larynx and hypopharynx experimentally are 1000-fold greater than those reported in the airways of patients with LPR, GERD and asthma, or lung disease (0.3–50 mM[96,103,104] versus 0.8–32uM[105–109]) and result in morphologic changes inconsistent with those of LPR patients such as cell membrane ‘blebbing’[110].

Pepsin is present in all refluxate[55]. Moreover, it is frequently detected in airway tissue and secretions from LPR patients but absent in MII-pH-confirmed reflux-free subjects, and thus may be predictive of reflux-attributed symptoms and disease[20,39,46,50,55,59,65,67,68,111,112]. Pepsin at 1mg/ml in the stomach is diluted by saliva as it is refluxed proximally. A range of concentrations have been reported in airways: 2.5μg/ml in saliva, 61.5μg/ml in nasal secretions[113,114] and 360μg/ml in middle ear fluid[115]. To model chronic LPR within a limited experimental timeframe, 300μg/ml was employed herein[1,77,116,117]. Pepsin-mediated damage and inflammatory changes reported *in vitro* and *in vivo*, including the histologic changes herein, are consistent with those observed in LPR patients[62–64,66,70,118–122].

Compelling evidence from multiple groups highlights a major role for pepsin, independent of gastric acid, in reflux-attributed laryngeal symptoms and findings refractory to PPI therapy.

While pepstatin is a potent pepsin inhibitor, its poor water-solubility and pharmacokinetic properties make it a suboptimal therapeutic candidate. Structural data herein indicated that inhibitor binding to the active cleft of pepsin is predominantly stabilized by van der Waals contacts, making rational design of inhibitors difficult. Testing existing inhibitors of other aspartic proteases was therefore deemed the most efficacious route for identification of a pepsin-targeting therapeutic.

There are currently ten commercially available HIV protease inhibitors.[123] Seven were amenable to testing in our *in vitro* binding and inhibition assays and four (amprenavir, ritonavir, saquinavir and darunavir) bound and inhibited pepsin with IC_{50} in the low micromolar range, validating our hypothesis that existing therapeutic protease inhibitors may exhibit anti-peptic activity. Two drugs were selected for *in vivo* study based on anti-peptic activity from *in vitro* assays, cost and reported side effects. While saquinavir exhibits known side effects and interactions (QT prolongation, heart block, high blood lipids and liver problems) and has high cost, amprenavir, ritonavir, and darunavir have minimal side effects (diarrhea, nausea and vomiting).[123] Darunavir is more costly than amprenavir and ritonavir, but had the lowest IC_{50} for pepsin. Darunavir, with the lowest IC_{50}, and fosamprenavir, a prodrug of amprenavir with improved bioavailability and favorable tolerability were therefore selected for assessment *in vivo*. Given that proximal reflux is inconsistent in surgical models of GER[124], we employed a model involving mechanical wounding and pepsin/acid instillation which reliably replicates epithelial alterations similar to that observed in patients with LPR[1,63,70,118,119,125]. Using this model, the human-equivalent dose of fosamprenavir, but not darunavir, prevented pepsin-mediated laryngeal damage. When administered locally by inhalation, treatment with either compound preserved normal laryngeal histology despite pepsin exposure.

As these data suggest, reformulation for inhaled, local delivery would be expected to improve drug efficacy and limit side effects. Preliminary computational fluid dynamics analysis (unpublished) revealed an optimal particle size of 9–12μm for deposition in the human larynx in agreement with previous studies[126].

The study herein was intended to investigate whether a pepsin inhibitor may prevent laryngeal damage caused by pepsin exposure *in vivo*. As with any experimental observation, caution should be exercised when translating *in vivo* findings from a limited number of animals to the clinical situation. Potential differences between mouse and human respiratory pathobiology should be kept in mind while evaluating the clinical implications of these data. Established methods for *in vivo* modeling of aerodigestive tract damage attributed to GERD and LPR[1,96–99] were utilized herein and demonstrated mucosal damage consistent with the clinical presentation of LPR supporting their use for assessing drug prevention of LPR-attributed injury: at the four-week conclusion of treatment, no mucosal damage was detectable given mechanical injury and neutral solvent, whereas multi-layered, reactive epithelia with apoptosis was observed in the pepsin and acid treatment groups.

The mouse epiglottis occupies a transitional zone from stratified squamous epithelium of the vocal fold to ciliated pseudostratified columnar epithelium at the supraglottis and infraglottis.[127] To avoid misinterpreting squamous epithelium of the vocal folds as signs of injury, representative images were collected rostral to vocal folds, exclusively from tissue with visible thyroid to serve as a guide. Additional features of reactive epithelia (darkened nuclei, variable nuclear diameter, and increased nuclear to cytoplasmic ratio, intraepithelial inflammatory cells, and apoptosis) in pepsin-treated groups, absent in control pH7.0 and those receiving fosamprenavir or darunavir, confirmed epithelial reactivity due to pepsin and the efficacy of HIV protease for prevention of pepsin-mediated injury. While these data are qualitative and would be bolstered by less subjective quantitative measures, the evidence herein provides initial proof-of-concept that a pepsin-targeting therapeutic may reduce mucosal damage akin to that observed in LPR patients and supports more in-depth investigation. Research is ongoing in our laboratory to examine fosamprenavir protection against pepsin-mediated changes in laryngeal cell viability and inflammatory and carcinogenic gene and protein expressions. Further research is also warranted to determine whether laryngeal protection by fosamprenavir aerosol *in vivo* was due to systemic activity or local conversion to amprenavir. The intestine is the primary site of fosamprenavir metabolism. Conversion of fosamprenavir to amprenavir by alkaline phosphatase (ALP), which is required for its transepithelial flux and subsequent metabolism by cytochrome P450 enzymes, has been shown to occur via intestinal ALP at or near the surface of Caco-2 cells.[128,129] It is possible, however, that inhaled fosamprenavir is converted to amprenavir in the airways by serum ALP, just as similar phosphate ester prodrugs are converted by sera collected from healthy subjects.[130] Inhaled fosamprenavir may also be converted by salivary ALP or that expressed by respiratory mucosa and immune cells recruited to tissue injury.[131,132] Given that ALP is elevated during inflammation[132–134] and carcinogenesis including that of the larynx to which LPR contributes,[10,74,135–137] ALP may be elevated in LPR-damaged airways thereby increasing fosamprenavir conversion at the desired site of activity. Drug formulations that prolong retention in the aerodigestive tract could further improve local drug conversion and topical activity. Research is ongoing in our laboratory to examine the efficiency of fosamprenavir conversion by laryngeal epithelium, saliva and sera and a dose-response study is underway in the *in vivo* mouse model to compare the relative efficacies of inhaled fosamprenavir and amprenavir against pepsin-mediated damage.

While additional experimental data will aid our understanding of laryngeal protection by fosamprenavir, LPR symptom improvement will be the ultimate determinant of a successful medical therapy. A randomized placebo-controlled trial therefore represents the best test of a therapeutic compound. Such a trial of fosamprenavir is feasible given that an oral formulation is FDA-approved and an a priori responder definition of clinically meaningful symptom improvement has been established per FDA guidelines.[138] Intriguingly, pilot epidemiological data (unpublished) support the therapeutic potential of HIV protease inhibitors for LPR and warrant follow-up: among 2,062 adult HIV patients prescribed an HIV protease inhibitor (Froedtert Memorial Lutheran Hospital, Milwaukee, WI, July 2014–2016; Medical College of Wisconsin Institutional Review Board, 13874) just 0.2% had documented LPR whereas the incidence in the general population is 10–34%.[139,140]

These data lend preliminary support for clinical investigation of fosamprenavir as a novel therapeutic approach for LPR.

CONCLUSION

Compelling evidence highlights a major role for pepsin (independent of gastric acid) in reflux-attributed laryngeal symptoms and endoscopic findings refractory to PPI therapy. Fosamprenavir and darunavir, FDA-approved retroviral therapies for HIV/AIDS, bind and inhibit pepsin, abrogating pepsin-mediated laryngeal inflammation and mucosal damage in an LPR mouse model. These drugs target a foreign virus so are ideal to repurpose, allowing a clinical trial to assess efficacy for a much-needed medical treatment for patients faster than could be achieved with novel compounds. Reformulation for local inhaled delivery could further improve outcomes and limit side effects.

Acknowledgments:

The authors would like to thank Leah Radde, Alexandra Dzubinsky, and Gino Scuncio for their preliminary work in this project.

Funding:

Work was funded by the Medical College of Wisconsin Therapeutics Accelerator Program, Department of Pharmacology, and Department of Otolaryngology and Communication Sciences. This work was also supported by the National Institutes of Health (R35GM128840 to B.C.S.).

Data Availability

Structural data are available in the Worldwide Protein Databank (accession codes 6XCY, 6XCT, 6XCZ, 6XD2; http://www.wwpdb.org/). Additional information is available from the corresponding author on reasonable request.

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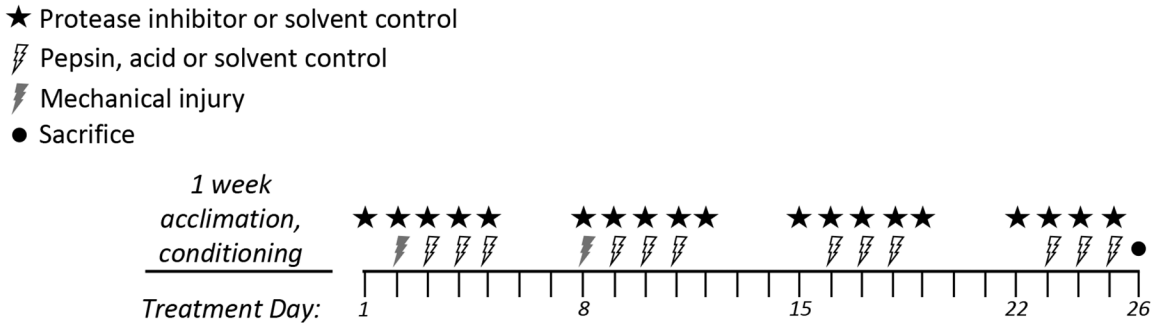

Figure 1.
Treatment schema of in vivo mouse study.

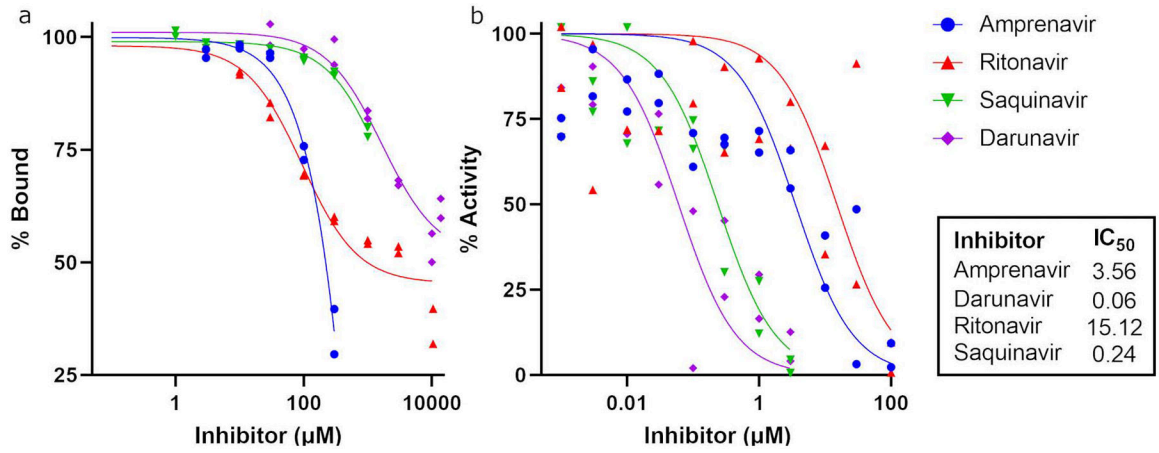

Figure 2.
Binding (a) and activity (b) curves of pepsin with HIV protease inhibitors.

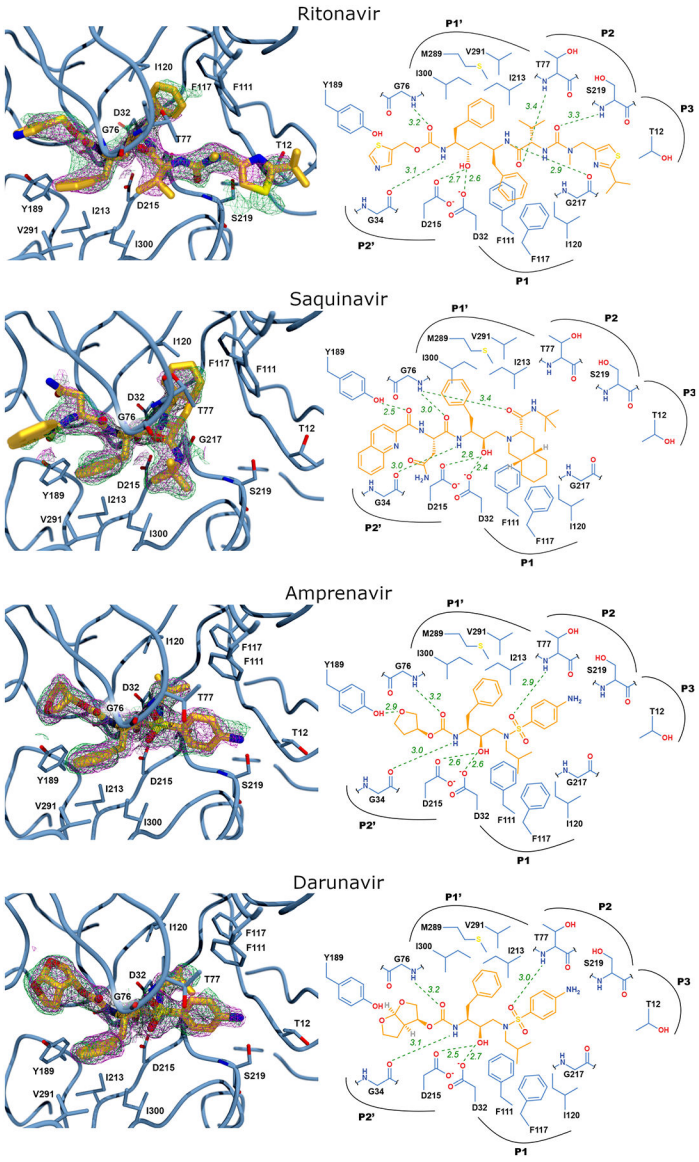

Figure 3. Pepsin and HIV protease inhibitor structural data.

Left panels: The active site of porcine pepsin with HIV protease inhibitor bound. The 2Fo-Fc electron density map contoured at 1.0σ is shown as magenta mesh and the 2Fo-Fc simulated annealing composite omit map, also contoured at 1.0σ, is shown as green mesh. Right panels: Schematic view of the active site with ritonavir bound showing potential hydrogen bonding interactions as green, dashed lines. Electron density maps were generated via POVSCRIPT and POV-Ray and schematic representation by MarvinSketch, and Adobe Illustrator.

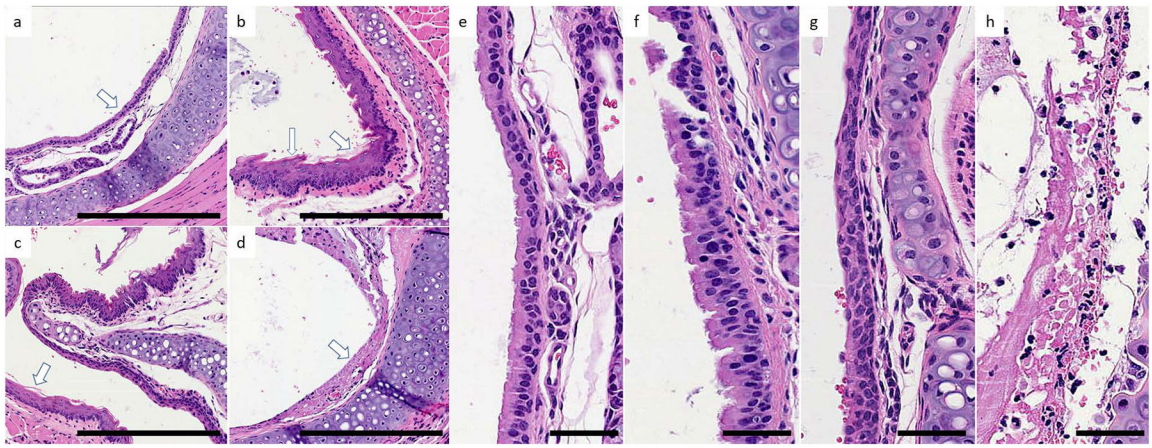

Figure 4. Laryngeal epithelial damage by pepsin and acid *in vivo*.

Representative specimens from treatment groups. Paired images at 40x (a-d) and 200x (e-h) magnification collected rostral to vocal folds, representing larynx: pH7 (a,e), pH4 (b,f), 0.3 mg/ml pepsin at pH7 (c,g), and 0.3 mg/ml pepsin at pH4 (d,h). (a,e) Normal respiratory columnar epithelium (arrow) about one cell layer thick with basal polarization of the nuclei and ciliated apical surfaces. (b,f) Reactive epithelium characterized by thickening (fat arrow) and focal squamous epithelia (long arrow) with loss of cilia. In other areas, relative thickening of the mucosa with moderately increased nuclear to cytoplasmic (N:C) ratio and irregular, condensed chromatin is seen. (c,g) Thickened respiratory epithelium with pseudostratification of the epithelial cells. Keratinization (arrow) is present in multiple foci. Significant increase in the N:C ratio with loss of nuclear polarization and reduction in the apical cilia is evident in several regions of this treatment group. (d,h) Respiratory epithelium is necrotic (arrow) and replaced by an inflammatory exudate. A brisk, acute inflammatory infiltrate infiltrates the submucosal area. Scale bars a-d = 100um; e-h = 50um.

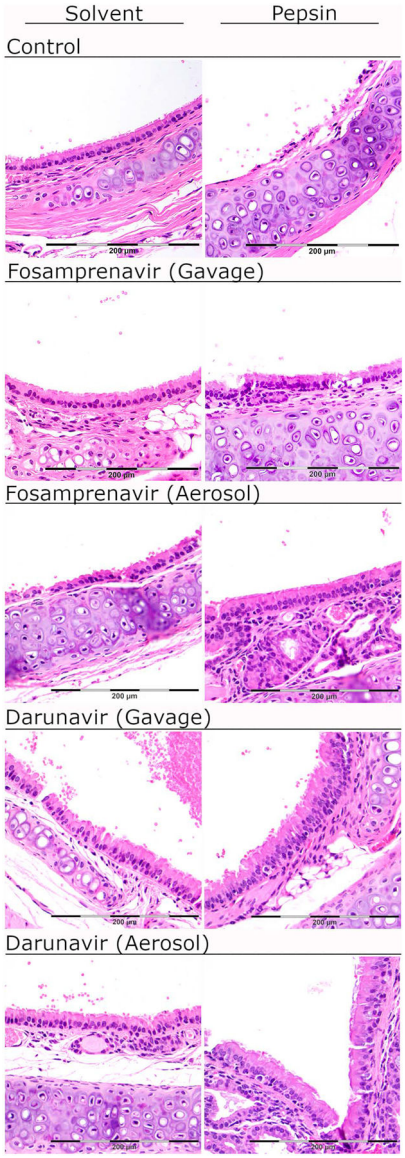

Figure 5. Fosamprenavir gavage and aerosol and darunavir aerosol prevent pepsin-mediated laryngeal damage *in vivo*.

Representative specimens at 400x. Solvent control group laryngeal epithelium was characterized by a single layer of respiratory epithelium with no reactive changes. In mice treated with pepsin-pH7, the laryngeal epithelium exhibited reactive epithelial changes and apoptotic debris. Fosamprenavir gavage and aerosol protected against pepsin-mediated laryngeal damage as indicated by normal histology in mice receiving fosamprenavir gavage or aerosol with saline (solvent), or fosamprenavir gavage or aerosol with pepsin-pH7. Darunavir gavage elicited mild reactivity (rare intraepithelial lymphocytes) in the saline treatment group; the darunavir gavage group with pepsin-pH7 appeared similar. Darunavir aerosol provided mild protection against pepsin-mediated damage. Epithelial injury was still present (mildly increased intraepithelial inflammatory cells and reactive epithelial cells), however no apoptosis was observed. Scale bar=200um.

Table 1.

Crystallographic data collection and model refinement statistics

	Pepsin·Amprenavir	Pepsin·Ritonavir	Pepsin·Darunavir	Pepsin·Saquinavir
PDB Entry	**6XCT**	**6XCY**	**6XD2**	**6XCZ**
Data collection				
Resolution (Å) (last shell)[a]	72.02 – 1.99 (2.04 – 1.99)	53.17 – 2.05 (2.11 – 2.05)	49.34 – 1.90 (1.97 – 1.90)	57.50 – 1.89 (1.93 – 1.89)
Space group	P 65 2 2	P 65 2 2	P 65 2 2	P 65 2 2
a, b, c (Å)	66.1, 66.1 288.1	66.2, 66.2, 285.5	66.2, 66.2, 290.0	66.4, 66.4, 284.6
α, β, γ (°)	90, 90, 120	90, 90, 120	90, 90, 120	90, 90, 120
R_{merge}[a]	0.057 (0.099)	0.10 (0.56)	0.088 (0.25)	0.091 (0.73)
R_{meas}[a]	0.060 (0.11)	0.13 (0.73)	0.092 (0.26)	0.110 (0.85)
R_{pim}[a]	0.021 (0.038)	0.084 (0.46)	0.026 (0.077)	0.054 (0.42)
$CC_{1/2}$[a]	0.999 (0.991)	0.989 (0.593)	0.995 (0.981)	0.995 (0.649)
No. of unique reflections[a]	26876 (1807)	23751 (1831)	30916 (2998)	30114 (1885)
Completeness (%)[a]	99.9 (99.5)	98.2 (99.8)	99.85 (99.90)	97.8 (98.0)
Multiplicity[a]	13.4 (12.8)	3.1 (3.2)	12.0 (11.8)	5.5 (5.9)
$\langle I/\sigma(I)\rangle$[a]	31.6 (18.8)	6.3 (2.3)	35.71 (10.34)	7.9 (1.6)
Model Refinement				
Reflections used in refinement[a]	26760 (2580)	23747 (2360)	30888 (2995)	30072 (2948)
Reflections used for R_{free}[a]	1312 (114)	1214 (116)	1574 (125)	1544 (145)
R_{cryst} (R_{free})[a]	0.1907 (0.1906)	0.2173 (0.2586)	0.1997 (0.1887)	0.2260 (0.2941)
Wilson B-factor (Å^2)	17.73	34.87	18.85	27.03
Average B factor (Å^2)	20.66	45.32	22.66	38.38
Protein atoms	19.62	44.98	21.61	37.97
Ligand atoms	24.29	71.10	26.63	59.65
Solvent	27.46	40.81	29.22	38.11
Root-mean-square (RMS) deviations				
Bond lengths (Å)	0.009	0.011	0.013	0.015
Bond angles (°)	0.77	1.48	1.02	1.68
Coordinate error (Å)[b]	0.14	0.12	0.15	0.10
Ramachandran statistics				
Favored/allowed/outliers (%)	99.37/0.32/0.32	97.82/1.87/0.31	99.37/0.32/0.32	98.13/1.56/0.31
Rotamer outliers (%)	0.00	1.82	0.00	2.92
Clashscore	0.85	2.72	2.33	2.93

[a]Values in parentheses apply to the high-resolution shell indicated in the resolution row

[b] Maximum-likelihood based estimates of coordinate error