Received: 11 May 2023 | Accepted: 12 June 2023

DOI: 10.1002/lio2.1102

ORIGINAL RESEARCH

Laryngoscope Investigative Otolaryngology

Amprenavir inhibits pepsin-mediated laryngeal epithelial disruption and E-cadherin cleavage in vitro

Tina L. Samuels MS[1] | Simon Blaine-Sauer BS[1] | Ke Yan PhD[2] | Nikki Johnston PhD[1,3]

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

[2]Department of Pediatrics Quantitative Health Sciences, Medical College of Wisconsin, Milwaukee, Wisconsin, USA

[3]Department of Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, Wisconsin, USA

Correspondence
Nikki Johnston, Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, 9200 W Wisconsin Ave, Milwaukee, WI 53226, USA.
Email: njohnston@mcw.edu

Funding information
Dr. Jamie Koufman; Medical College of Wisconsin; Mr. Eric Becker and family

Abstract

Background: Laryngopharyngeal reflux (LPR) causes chronic cough, throat clearing, hoarseness, and dysphagia and can promote laryngeal carcinogenesis. More than 20% of the US population suffers from LPR and there is no effective medical therapy. Pepsin is a predominant source of damage during LPR which disrupts laryngeal barrier function potentially via E-cadherin cleavage proteolysis and downstream matrix metalloproteinase (MMP) dysregulation. Fosamprenavir (FDA-approved HIV therapeutic and prodrug of amprenavir) is a pepsin-inhibiting LPR therapeutic candidate shown to rescue damage in an LPR mouse model. This study aimed to examine amprenavir protection against laryngeal monolayer disruption and related E-cadherin proteolysis and MMP dysregulation in vitro.

Methods: Laryngeal (TVC HPV) cells were exposed to buffered saline, pH 7.4 or pH 4 ± 1 mg/mL pepsin ± amprenavir (10–60 min). Analysis was performed by microscopy, Western blot, and real time polymerase chain reaction (qPCR).

Results: Amprenavir (1 μM) rescued pepsin acid-mediated cell dissociation ($p < .05$). Pepsin acid caused E-cadherin cleavage indicative of regulated intramembrane proteolysis (RIP) and increased *MMP-1,3,7,9,14* 24-h postexposure ($p < .05$). Acid alone did not cause cell dissociation or E-cadherin cleavage. Amprenavir (10 μM) protected against E-cadherin cleavage and *MMP-1,9,14* induction ($p < .05$).

Conclusions: Amprenavir, at serum concentrations achievable provided the manufacturer's recommended dose of fosamprenavir for HIV, protects against pepsin-mediated cell dissociation, E-cadherin cleavage, and MMP dysregulation thought to contribute to barrier dysfunction and related symptoms during LPR. Fosamprenavir to amprenavir conversion by laryngeal epithelia, serum and saliva, and relative drug efficacies in an LPR mouse model are under investigation to inform development of inhaled formulations for LPR.

KEYWORDS

antireflux medication, epithelial barrier function, extraesophageal reflux, laryngopharyngeal reflux, medical therapy

1 | INTRODUCTION

Laryngopharyngeal reflux (LPR), an inflammatory condition of the upper aerodigestive tract arising from direct and indirect effects of gastroduodenal reflux, is estimated to affect more than 20% of the US population and contribute to 10% visits to otolaryngologists.[1–3] The most prevalent symptoms are globus, hoarseness, cough, throat clearing, and postnasal drip.[3] A distinct clinical entity from gastroesophageal reflux disease (GERD), fewer than 50% exhibit classical GERD symptoms (heartburn and regurgitation).[3,4] LPR has a significant impact on quality of life and contributes to serious and life-threatening illness including airway stenosis, reactive airway disease, upper airway dysplasia, and carcinogenesis.[5] LPR incurs high cost to patient and society given its typical recurrence and requirement for prolonged treatment. The economic burden of LPR is >$52 billion per year, 5.6-fold greater than that of GERD, with more than half the cost attributed to proton pump inhibitors (PPIs).[6,7]

Although acid suppressing PPIs are the first-line therapy for GERD, their use for LPR is controversial in light of studies failing to demonstrate superiority to placebo and failure of the drugs to provide benefit even at exceedingly high dose.[8,9] Roughly 40% of LPR cases fail to achieve partial symptomatic improvement with PPI therapy and complete resolution is rare.[8,10,11] Combined multichannel intraluminal impedance-pH (MII-pH) monitoring has demonstrated that many episodes of LPR are weakly to nonacidic and that these episodes are associated with persistent symptoms in acid-suppressed patients.[12–16] Studies suggest that PPI recalcitrant symptoms are alleviated by more comprehensive therapeutic strategies that reduce reflux occurrence or target constituents beyond acid (e.g., antireflux surgery,[17–22] dietary and lifestyle modification,[23–25] and alginate[26]) indicating that nonacid constituents of refluxate play a significant role in the pathophysiology of LPR.

A growing body of evidence implicates the gastric enzyme pepsin as a predominant damaging element of LPR.[27] Pepsin is frequently detected in airway tissue and secretions from LPR patients yet absent in MII-pH-confirmed reflux-free subjects.[28–31] Its abundance and correlation with cancer-associated changes such as increased interleukin-8 and DNA damage in dysplastic or tumor airway tissue support its contribution to the elevated risk of laryngeal cancer among LPR patients.[5,32–37] In experimental models, pepsin leads to inflammatory and carcinogenic changes irrespective of pH in vitro and in vivo including altered transcriptomic profiles; promotion of apoptotic resistance, cell migration, anchorage-independent growth and glycolysis; and development of tumors in a hamster cheek model.[27,33,35–41] Although at neutral pH, pepsin is transiently inactivated and taken up by respiratory epithelia by receptor-mediated endocytosis where it appears to exert its proinflammatory and carcinogenic effects by initiation of molecular signaling events or intracellular reactivation,[27] pepsin in strong to weak acid (up to pH 6.5) retains enzymatic activity[42] and may degrade cell adhesion molecules resulting in impaired epithelial barrier integrity,[43–45] a hallmark of LPR and GERD thought to contribute to symptom origination.[46,47] The adhesion molecule E-cadherin is inversely correlated with pepsin in LPR[48] and has been demonstrated to undergo regulated intramembrane proteolysis (RIP) in GERD and LPR specimens, releasing biologically active fragments known to promote proinflammatory and cancer-associated signaling, including regulation of tissue-remodeling matrix metalloproteinases (MMPs).[49–53]

We previously reported that the HIV protease inhibitor, amprenavir, bound and inhibited pepsin at micromolar concentrations.[54] An oral solution of its prodrug, fosamprenavir, prevented laryngeal damage in a mouse model of LPR when administered at a dose equivalent to that provided HIV patients. Herein, we sought to utilize a recently established in vitro model of laryngeal epithelial barrier disruption by acidified pepsin[43] to investigate the efficacy of amprenavir to prevent peptic disruption of cell monolayers and its potential role in E-cadherin RIP and downstream MMP misregulation.

2 | METHODS

2.1 | Cell culture

Immortalized laryngeal true vocal cord epithelial cells[41] (TVC HPV) were cultured in Small Airway Epithelial Cell Growth Media (Promocell, Heidelberg, Germany) with 1× Antibiotic-Antimycotic (ThermoFisher Scientific, Waltham, MA) as previously[43] in triplicate wells to 100% confluence for examining cell dissociation, 90% for protein expression and 50% for gene expression.

To examine rescue of pepsin-mediated cell dissociation, cultures were pretreated for 1 h in Hank's buffered saline solution (HBSS; pH 7.4 unless otherwise stated) ±1–10 μM fosamprenavir (Anant Laboratories, Ambernath, India) or 0.5% dimethyl sulfoxide (DMSO; vehicle). Cultures were washed twice in HBSS and treated in HBSS or HBSS pH 4 ±1 mg/mL porcine pepsin (Sigma-Aldrich, St. Louis, MO) ±1–10 μM fosamprenavir, 1–10 μM amprenavir (Sigma-Aldrich), or DMSO at 37°C/5% CO_2 for 1 h and assessed immediately by light microscopy.

Treatment with HBSS or HBSS pH 4 ± 1 mg/mL porcine pepsin ±10–100 μM amprenavir or DMSO for 10–30 min was used to investigate rescue of E-cadherin RIP by Western blot or immunofluorescence following immediate harvest or fixation.

Cells were treated at the same pH and dose for 15 min, rinsed twice in HBSS, and allowed to rest in normal growth media for 24 h prior to harvest and assessment of MMP expression by qPCR.

The role of MMPs in E-cadherin RIP and MMP expression were tested by 2-h pretreatment with HBSS ±20 μM GM6001 (broad-spectrum MMP inhibitor, Sigma-Aldrich) or 0.5% DMSO prior to treatment in HBSS or HBSS pH 4 ±1 mg/mL porcine pepsin ±20 μM GM6001 for 30 min (Western blot and immunofluorescence) or 15 min (qPCR; as described above, treatment was followed by two washes in HBSS and 24 h rest in normal growth media prior to harvest). The selected concentration of GM6001 was previously shown to prevent E-cadherin cleavage and related changes in cell adhesion and migration in vitro.[55] Half-maximal inhibitory concentration values reported for GM6001 against human MMPs are: MMP-1 (6 nmol/L), MMP-2 (7/17 nmol/L), MMP-3 (28 nmol/L), MMP-7 (41 nmol/L), MMP-8 (1.4 nmol/L),

MMP-9 (4.1/15 nmol/L), MMP-12 (23 nmol/L), MMP-13 (3.2 mmol/L), MMP-14 (23/33 nmol/L), MMP-15 (6 nmol/L), MMP-16 (8 nmol/L), and MMP-26 (17 nmol/L); and inhibition of MMP-10, MMP-17, MMP-20, MMP-21, TACE, ADAM19, other ADAMs, anthrax lethal factor, neprilysin, leucine aminopeptidase, and DPPIII has also been reported.[56–58]

2.2 | Cell dissociation

After two washes in HBSS, images were obtained on an inverted microscope (Nikon, Tokyo, Japan and Metamorph Inc., Nashville, TN). The percent cell-free area of a single image from each well ($n = 3$) was quantified using PHANTAST[59] plug-in for FIJI.[60]

2.3 | Western blot

Cells were harvested in cold radio–immunoprecipitation assay (RIPA) lysis buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris-Cl, pH 7.4) containing protease inhibitor. Proteins were separated (4%–20% TGX, Bio Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride. Membranes were blocked (5% milk and 0.1% Tween-20 in phosphate buffered saline, PBS) and probed with E-cadherin C-terminal (4A2C7, ThermoFisher Scientific, per Jovov et al.[51]), ADAM-10 (AB19026, Sigma-Aldrich), actin (CP01, Sigma-Aldrich), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Agilent Technologies, Santa Clara, CA). Densitometry was performed using FIJI (version 1.52a, National Institutes of Health, Bethesda, MD); signal corresponding to full-length E-cadherin and its fragments in each lane was normalized to actin signal from the same lane and mean of three lanes representing biological triplicate compared across groups.

2.4 | Immunofluorescence

Cells were fixed in paraformaldehyde, immunostained as previously[61] in anti-E-cadherin (HECD1, ThermoFisher Scientific per Jovov et al.[51]) and mounted with DAPI (ThermoFisher Scientific).

2.5 | Real time qPCR

RNA was extracted via TRIZOL and purity and concentration assessed by UV spectroscopy (Nanodrop 2000; ThermoFisher Scientific). RNA was reverse transcribed using Superscript IV VILO (ThermoFisher Scientific). qPCR was performed in quadruplicate reactions using Taqman gene expression assays in a Viia7 instrument (ThermoFisher Scientific) per manufacturer's instructions. Threshold cycle (Ct) values <36 were used for analysis, and gene expression was normalized to housekeeping gene *HPRT1*.

2.6 | Statistical analysis

Student's *t*-test was used to compare groups. For qPCR, RQs were calculated via delta–delta method. Log transformation was applied to cell dissociation data due to skewness of data. $p < .05$ was considered significant.

3 | RESULTS

Pepsin pH 4 elicited cell dissociation which was rescued by ≥1 μM amprenavir ($p < .05$) in a dose-dependent manner (Figure 1 and Supplementary Table S1); acid alone did not induce cell dissociation. Rescue by 10 μM amprenavir was complete as indicated by non-significant difference relative to Control confluence ($p = .089$). Rescue of cell dissociation by ≥10 μM fosamprenavir ($p < .05$) suggested prodrug conversion by laryngeal epithelial cells.

Amprenavir exclusively was used for mechanistic studies. Western blot demonstrated that pepsin pH 4 caused near total cleavage of full-length E-cadherin by 30 min ($p = .0083$) while acid (pH 4) alone for 30 min did not cause E-cadherin RIP (Figure 2 and Tables S2 and S3). Depletion of intact E-cadherin by pepsin pH 4 was partially rescued by 10 μM amprenavir ($p = .0002$) and rescued to a lesser degree by 20 μM GM6001 ($p = .0045$). E-cad/CTF1 and CTF2 were not detected in Control or pH 4 groups but were present following treatment with pepsin pH 4; peptic induction of CTF1 was partially rescued by 10 μM amprenavir or 20 μM GM6001 ($p < .05$). Membrane-localized E-cadherin staining appeared depleted or more diffuse given treatment with pepsin pH 4 (but not pH 4 alone) and was partially rescued by amprenavir (Figure 3). Membrane-associated staining was not completely abolished by pepsin pH 4 as would be expected based on results of Western blot: this may be attributed to confounding by a proportion of E-cadherin N-terminal cleavage fragment (NTF/sE-cad) that remains membrane-bound through interaction with transmembrane proteins.[53]

Pepsin pH 4 increased *MMP-1,3,7,9,14* expression 24 h postexposure ($p < .05$; Table 1) but did not induce *MMP2*, whereas acid alone caused significant upregulation of exclusively *MMP14* ($p < .05$). Amprenavir (10 μM) partially rescued *MMP-1,9,14* induction and 100 μM amprenavir partially rescued *MMP7* ($p < .05$). GM6001 rescued exclusively *MMP7* ($p < .05$).

4 | DISCUSSION

As research over the last 20 years has highlighted the limited utility of acid-targeting approaches to LPR and identified pepsin as a biomarker and key determinant of LPR pathophysiology, considerable interest has developed in pepsin as a therapeutic target. We recently identified an HIV protease inhibitor (amprenavir, commonly administered as fosamprenavir) which binds and inhibits pepsin in the nanomolar range and rescued histologic indications of peptic injury in a mouse

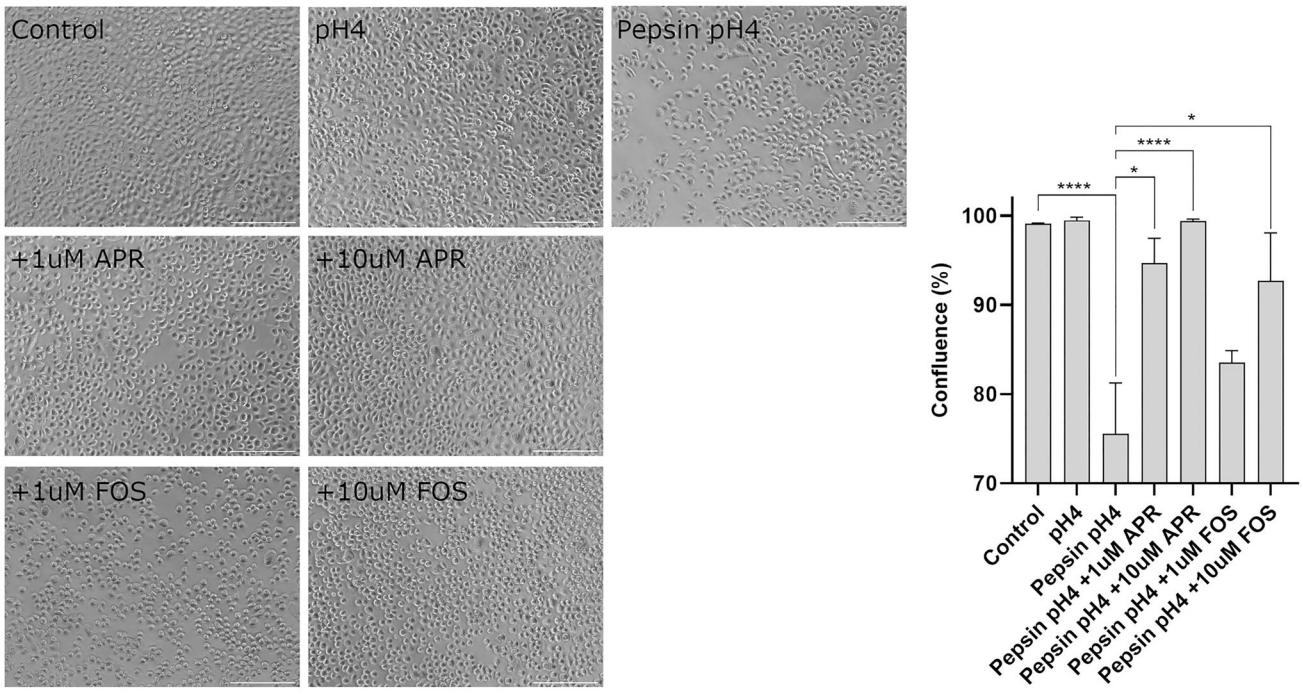

FIGURE 1 Pepsin-acid (1 mg/mL pepsin pH 4, 1 h) induced cell dissociation and rescue. Cell dissociation was rescued by 1 μM amprenavir (APR) or 10 μM fosamprenavir (FOS), $p < .05$. Scale bar = 100 μm; error bars = SD; $^{*}p < .05$; $^{****}p < .0001$.

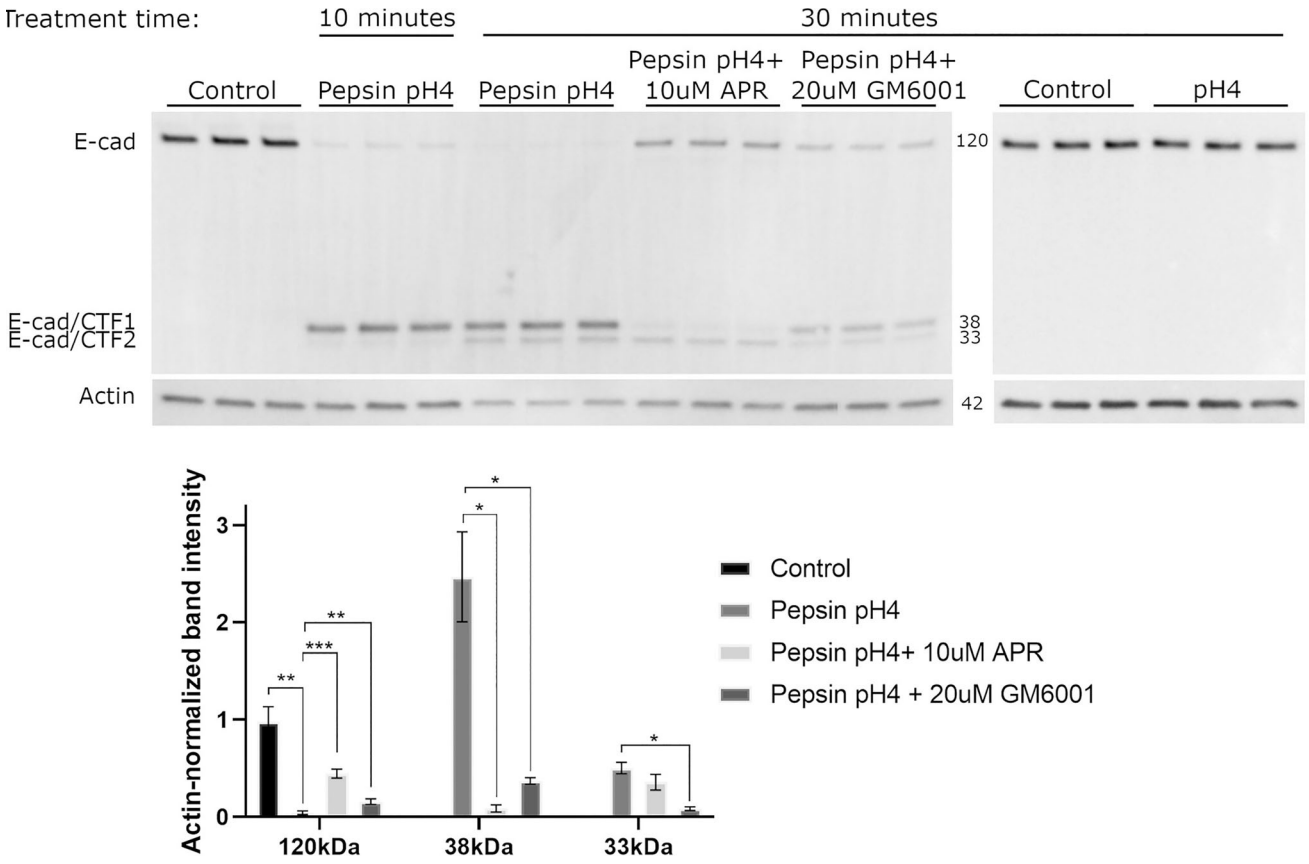

FIGURE 2 Pepsin-acid (1 mg/mL pepsin pH 4) cleaved nearly all full-length E-cadherin (E-cad; 120 kDa) by 30 min resulting in 38 and 33 kDa C-terminal E-cad/CTF1 and E-cad/CTF2 fragments indicative of regulated intramembrane proteolysis (RIP). Amprenavir (APR) co-treatment or broad-spectrum matrix metalloproteinase (MMP) inhibitor (GM6001, pre and co-treatment) partially rescued pepsin-acid mediated E-cadherin cleavage. Acid alone did not induce E-cadherin cleavage. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

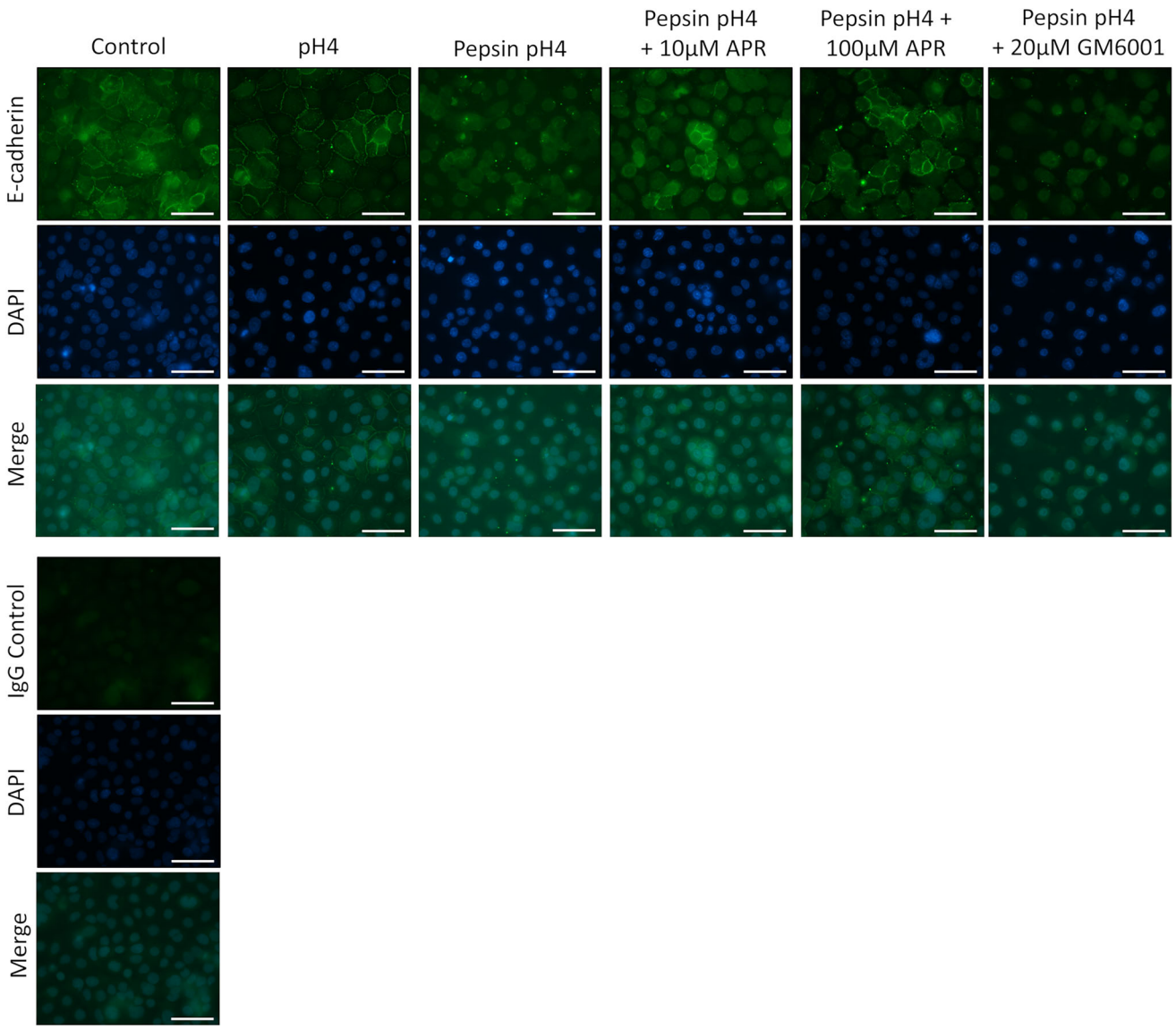

FIGURE 3 Pepsin-acid (1 mg/mL pepsin pH 4, 30 min) caused depletion of membrane localized E-cadherin which was partially rescued by the pepsin inhibitor, amprenavir (APR), but not the broad-spectrum matrix metalloproteinase (MMP) inhibitor, GM6001.

LPR model.[54] Many LPR episodes are weakly acidic and weakly acidic pepsin elicits microscopically evident cell dissociation in vitro[43] akin to dilated intercellular spaces which are a hallmark of LPR and GERD. Cell dissociation in vitro is not incurred by acid alone[43] in agreement with studies in animal models finding that pepsin or bile salts are required to produce histologic injury and sustained epithelial barrier disruption in vivo at pH >2.[45,62–64] The acute erosive activity of acidified pepsin has historically been attributed to digestion of protein constituents of the mucin layer, basement membrane, and intercellular junctions.[44,45]

E-cadherin is the primary protein constituent of adherens junctions. It plays a crucial role in ensuring tissue integrity by immobilizing cells within the epithelium, facilitating contact inhibition of growth, and maintaining proper functioning of tight junctions, the primary permeability barrier of the epithelium. E-cadherin also regulates cell migration and proliferation through interaction with catenins and growth factor signaling pathways. E-cadherin cleavage fragments present in LPR and GERD specimens, absent in reflux-free controls, suggest its RIP plays a role in the pathophysiology of reflux-attributed disease.[50,51] RIP is a relatively recently discovered phenomenon which releases latent signaling molecules through sequential extracellular and intramembrane (or intracellular) cleavage of transmembrane proteins by a sheddase and γ-secretase, respectively, yielding fragments that are frequently implicated in inflammatory and cancer-related signaling.[65] E-cadherin RIP produces an 80 kDa N-terminal fragment (soluble E-cadherin, sE-cad) which diffuses into the extracellular environment and may disrupt pairing of full-length E-cadherin dimers; chemoattract migrating cells; promote proliferation and apoptotic resistance via growth factor receptor signaling; and upregulate MMPs which facilitate cell migration and further E-cadherin RIP.[52,53]

TABLE 1 MMP gene expression 24-h posttreatment.

	Mean RQ ± SD	*p* rel Control	*p* rel Pepsin pH 4
MMP1			
Control	1.00 ± 0.02		
pH 4	1.11 ± 0.09	.11	.0077
1 mg/mL pepsin pH 4	4.84 ± 0.61	.0082	
10 μM APR + pepsin pH 4	3.60 ± 0.23	.0024	.029*
100 μM APR + pepsin pH 4	3.25 ± 0.67	.028	.038*
20 μM GM6001 + pepsin pH 4	4.67 ± 0.61	.0091	.75
MMP2			
Control	1.00 ± 0.25		
pH 4	1.10 ± 0.04	.57	<.0001
1 mg/mL pepsin pH 4	1.56 ± 0.02	.062	
10 μM APR + pepsin pH 4	1.20 ± 0.01	.30	<.0001*
100 μM APR + pepsin pH 4	1.01 ± 0.01	.96	<.0001*
20 μM GM6001 + pepsin pH 4	1.62 ± 0.09	.017	.33
MMP3			
Control	1.00 ± 0.15		
pH 4	1.11 ± 0.15	.41	.025
1 mg/mL pepsin pH 4	6.86 ± 1.63	.024	
10 μM APR + pepsin pH 4	4.43 ± 0.53	.0004	.070
100 μM APR + pepsin pH 4	4.22 ± 1.14	.038	.083
20 μM GM6001 + pepsin pH 4	5.73 ± 0.51	.0001	.32
MMP7			
Control	1.00 ± 0.42		
pH 4	0.82 ± 0.36	.60	.0026
1 mg/mL pepsin pH 4	5.59 ± 1.18	.0032	
10 μM APR + pepsin pH 4	3.52 ± 0.73	.0066	.061
100 μM APR + pepsin pH 4	2.96 ± 0.88	.025	.036*
20 μM GM6001 + pepsin pH 4	3.37 ± 0.55	.0041	.042*
MMP9			
Control	1.00 ± 0.16		
pH 4	1.14 ± 0.08	.23	.016
1 mg/mL pepsin pH 4	3.51 ± 0.55	.0016	
10 μM APR + pepsin pH 4	2.05 ± 0.26	.0040	.014*
100 μM APR + pepsin pH 4	1.36 ± 0.17	.052	.0030*
20 μM GM6001 + pepsin pH 4	3.80 ± 0.71	.0026	.61
MMP14			
Control	1.00 ± 0.11		
pH 4	1.40 ± 0.18	.032	.025
1 mg/mL pepsin pH 4	2.01 ± 0.25	.0031	
10 μM APR + pepsin pH 4	1.46 ± 0.07	.0039	.021*
100 μM APR + pepsin pH 4	1.14 ± 0.02	.10	.026*
20 μM GM6001 + pepsin pH 4	2.22 ± 0.29	.0025	.40

Abbreviations: APR, amprenavir; MMP, matrix metalloproteinase; RQ, relative quantity.
*$p < .05$, rescue relative to pepsin pH 4.

The integral membrane C-terminal fragment (38 kDa E-cad/CTF1) is then cleaved by a γ-secretase, releasing 33-kDa E-cad/CTF2 to the cytosol.[53] This liberates β-catenin which facilitates oncogenic Wnt signaling, and p120 catenin which promotes E-cad/CTF2 DNA-binding and apoptotic resistance via Kaiso.[53] Serum sE-cad is associated with cancer[66] and E-cadherin depletion is associated with

pepsin presence in LPR and GERD/LPR-attributed metaplasia and cancer.[37,67–69] E-cadherin is also depleted in other aerodigestive conditions associated with impaired barrier integrity.[70]

Herein, weakly acidic pepsin induced cleavage of nearly all full-length E-cadherin within 30 min which was partially rescued by the pepsin inhibitor, amprenavir. Although Jovov et al. speculated that refluxed acid caused E-cadherin cleavage in GERD biopsies via ADAM10 activation,[51] acid alone did not elicit E-cadherin RIP herein. Where acid alone has been shown to elicit E-cadherin cleavage in aerodigestive tract epithelia, the effect is delayed, observed after 24 h, and secondary to transcriptional regulation of MMPs.[49,50,71] While a number of epithelial E-cadherin sheddases would be present in our in vitro model (e.g., MMP-3,7,9,14,15 and ADAM-10,12,15)[53] most or all would be inhibited by GM6001.[56–58] Incomplete rescue of E-cadherin cleavage by GM6001 and partial rescue by the pepsin-inhibitor amprenavir herein support pepsin as an E-cadherin sheddase during LPR.

MMPs which are regulated by E-cadherin RIP and implicated in reflux-attributed disease were also assessed herein. *MMP-2,9,14* may be dysregulated by sE-cad[52] and *MMP7* by E-cad/CTF-related Wnt signaling.[72] MMP-9 mediates pepsin-induced mucin 5AC expression in airway epithelial cells contributing to hypersecretion and inflammation,[73] is induced by TGF-β signaling in tracheal cells, a key pathway in laryngotracheal stenosis,[74] is elevated in mild and severe GERD,[75] and plays a role in the early inflammatory response in a model of Barrett's esophagus (BE).[76] MMP-2, 7 and 9 have been implicated in laryngeal cancer.[77] MMP-1 is a pre-invasive factor for BE associated with GERD severity and esophageal adenocarcinoma (EAC).[78,79] MMP-14 is considered the "master MMP" given its role in activation of many MMPs and strong association with invasion, migration and angiogenesis of aerodigestive tract cancers.[80] Herein, pepsin acid-induced *MMP-1,3,7,9,14* expression 24-h postexposure; all but *MMP3* were partially rescued by amprenavir. The protective benefits of amprenavir against E-cadherin RIP and MMP dysregulation, which are in turn associated with tumorigenesis, metastasis, and poor cancer prognosis, have recently been corroborated in an esophageal culture model,[81] and are particularly intriguing given failure of the current mainstay treatment for severe GERD to demonstrate chemopreventive benefit or stem the rising incidence of EAC despite widespread use.[82]

The concentration of amprenavir shown protective herein is similar to the serum concentration achievable provided the manufacturer recommended dose for treatment of HIV.[83] Local delivery by aerosolization would permit lower dosing and limit the potential for side effects. Locally delivered fosamprenavir may be converted by alkaline phosphatase present in saliva, serum and epithelial cells as supported by conversion of other phosphate ester prodrugs[84,85] and fosamprenavir protection herein. Fosamprenavir conversion by saliva, serum, and epithelial cells and the relative efficacies of aerosolized amprenavir and fosamprenavir in an LPR mouse model are under investigation to inform the design of a dry powder inhaler for local delivery to the airways.

Limitations of this study include its basis on a single cell culture model. While cell lines are invaluable to scientific investigation and HPV E6/7-immortalized TVC HPV cells retain characteristics of the tissue of origin (cytokeratin expression, tight junctions, anchorage-dependent growth) supporting their use as a model,[41] caution should be exercised when extrapolating in vitro findings to clinical situations. Future experiments in the LPR mouse model developed in our laboratory would be useful to corroborate these observations. The results of the present study and previous report of fosamprenavir rescue of pepsin-mediated histologic damage in the LPR mouse model provide proof-of-concept for the use of fosamprenavir as a novel therapeutic for LPR. A 12-week randomized, double-blind, placebo-controlled clinical trial has been approved to assess the efficacy of an oral solution of fosamprenavir for LPR. If effective, an aerosolized drug for local inhaled delivery could further improve outcomes and limit side effects.

5 | CONCLUSIONS

LPR is a common illness with an adverse impact on quality of life and life-threatening complications including cancer. Acid suppressing therapies have proven ineffective for the treatment of LPR in agreement with evidence implicating pepsin as a major aggressor. Herein, the pepsin inhibitor amprenavir, at a concentration achievable given the manufacturer recommended dose for treatment of HIV, protected against peptic cell dissociation, E-cadherin cleavage and MMP induction in laryngeal cells which are associated with epithelial barrier disruption, inflammatory tissue remodeling and tumorigenesis. Protection by fosamprenavir herein suggests conversion of the prodrug by laryngeal epithelium and supports the potential of its local aerosolized delivery to allow for lower dosing and reduced likelihood of side effects. These data provide proof-of-concept supporting a clinical trial for the efficacy of fosamprenavir as a novel therapeutic for LPR.

ACKNOWLEDGMENT

The authors would like to thank Dr. Jamie Koufman and Mr. Eric Becker and his family for kind donations that supported this work.

CONFLICT OF INTEREST STATEMENT

This study was funded by the Medical College of Wisconsin Department of Otolaryngology and Communication Sciences and kind donations of Dr. Jamie Koufman and Mr. Eric Becker and his family. Nikki Johnston is co-founder, Chief Scientific Officer and an investor in N-Zyme Biomedical. Nikki Johnston is an inventor on International Patent Application PCT/US2021/027758, Aerosolized formulations of HIV protease inhibitors for the treatment of airway reflux, filed April 16, 2021, and US Patent Application 63/392,929, Sustained-release oral fosamprenavir for the treatment of reflux, filed July 28, 2022. Tina L. Samuels is an investor in N-Zyme Biomedical. Other authors have no financial relationships or conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Data herein were presented January 26–28, 2023 at the Triological Society Combined Sections Meeting, Coronado, CA USA.

ORCID

Tina L. Samuels https://orcid.org/0000-0002-7045-357X

Simon Blaine-Sauer https://orcid.org/0000-0001-6027-1409

REFERENCES

1. Altman KW, Stephens RM, Lyttle CS, Weiss KB. Changing impact of gastroesophageal reflux in medical and otolaryngology practice. *Laryngoscope*. 2005;115:1145-1153.
2. Reulbach TR, Belafsky PC, Blalock PD, Koufman JA, Postma GN. Occult laryngeal pathology in a community-based cohort. *Otolaryngol Head Neck Surg*. 2001;124:448-450.
3. Lechien JR, Akst LM, Hamdan AL, et al. Evaluation and management of laryngopharyngeal reflux disease: state of the art review. *Otolaryngol Head Neck Surg*. 2019;160:762-782.
4. Habermann W, Schmid C, Neumann K, Devaney T, Hammer HF. Reflux symptom index and reflux finding score in otolaryngologic practice. *J Voice*. 2012;26:e123-e127.
5. Parsel SM, Wu EL, Riley CA, McCoul ED. Gastroesophageal and laryngopharyngeal reflux associated with laryngeal malignancy: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol*. 2019;17:1253-1264 e1255.
6. Francis DO, Rymer JA, Slaughter JC, et al. High economic burden of caring for patients with suspected extraesophageal reflux. *Am J Gastroenterol*. 2013;108:905-911.
7. Gelardi M, Ciprandi G. Focus on gastroesophageal reflux (GER) and laryngopharyngeal reflux (LPR): new pragmatic insights in clinical practice. *J Biol Regul Homeost Agents*. 2018;32:41-47.
8. Lechien JR, Mouawad F, Barillari MR, et al. Treatment of laryngopharyngeal reflux disease: a systematic review. *World J Clin Cases*. 2019;7:2995-3011.
9. Portnoy JE, Gregory ND, Cerulli CE, et al. Efficacy of super high dose proton pump inhibitor administration in refractory laryngopharyngeal reflux: a pilot study. *J Voice*. 2014;28:369-377.
10. Lechien JR, Muls V, Dapri G, et al. The management of suspected or confirmed laryngopharyngeal reflux patients with recalcitrant symptoms: a contemporary review. *Clin Otolaryngol*. 2019;44:784-800.
11. Friedman M, Maley A, Kelley K, et al. Impact of pH monitoring on laryngopharyngeal reflux treatment: improved compliance and symptom resolution. *Otolaryngol Head Neck Surg*. 2011;144:558-562.
12. Sharma N, Castell DO. Further comment on proton pump inhibitor failures. *Clin Gastroenterol Hepatol*. 2009;7:363.
13. Tamhankar AP, Peters JH, Portale G, et al. Omeprazole does not reduce gastroesophageal reflux: new insights using multichannel intraluminal impedance technology. *J Gastrointest Surg*. 2004;8:890-897. discussion 897–898.
14. Tutuian R, Mainie I, Agrawal A, Adams D, Castell DO. Nonacid reflux in patients with chronic cough on acid-suppressive therapy. *Chest*. 2006;130:386-391.
15. Tutuian R, Vela MF, Hill EG, Mainie I, Agrawal A, Castell DO. Characteristics of symptomatic reflux episodes on acid suppressive therapy. *Am J Gastroenterol*. 2008;103:1090-1096.
16. Mainie I, Tutuian R, Shay S, et al. Acid and non-acid reflux in patients with persistent symptoms despite acid suppressive therapy: a multicentre study using combined ambulatory impedance-pH monitoring. *Gut*. 2006;55:1398-1402.
17. Falk GL, Van der Wall H, Burton L, Falk MG, O'Donnell H, Vivian SJ. Fundoplication for laryngopharyngeal reflux despite preoperative dysphagia. *Ann R Coll Surg Engl*. 2017;99:224-227.
18. Iqbal M, Batch AJ, Spychal RT, Cooper BT. Outcome of surgical fundoplication for extraesophageal (atypical) manifestations of gastroesophageal reflux disease in adults: a systematic review. *J Laparoendosc Adv Surg Tech A*. 2008;18:789-796.
19. Klimara MJ, Randall DR, Allen J, Figueredo E, Johnston N. Proximal reflux: biochemical mediators, markers, therapeutic targets, and clinical correlations. *Ann NY Acad Sci*. 2020;1481:127-138.
20. Lechien JR, Dapri G, Dequanter D, et al. Surgical treatment for laryngopharyngeal reflux disease: a systematic review. *JAMA Otolaryngol Head Neck Surg*. 2019;145:655-666.
21. Sidwa F, Moore AL, Alligood E, Fisichella PM. Surgical treatment of extraesophageal manifestations of gastroesophageal reflux disease. *World J Surg*. 2017;41:2566-2571.
22. Zhang C, Hu ZW, Yan C, et al. Nissen fundoplication vs proton pump inhibitors for laryngopharyngeal reflux based on pH-monitoring and symptom-scale. *World J Gastroenterol*. 2017;23:3546-3555.
23. Giacchi RJ, Sullivan D, Rothstein SG. Compliance with anti-reflux therapy in patients with otolaryngologic manifestations of gastroesophageal reflux disease. *Laryngoscope*. 2000;110:19-22.
24. Zalvan CH, Hu S, Greenberg B, Geliebter J. A comparison of alkaline water and Mediterranean diet vs proton pump inhibition for treatment of laryngopharyngeal reflux. *JAMA Otolaryngol Head Neck Surg*. 2017;143:1023-1029.
25. Koufman JA. Low-acid diet for recalcitrant laryngopharyngeal reflux: therapeutic benefits and their implications. *Ann Otol Rhinol Laryngol*. 2011;120:281-287.
26. McGlashan JA, Johnstone LM, Sykes J, Strugala V, Dettmar PW. The value of a liquid alginate suspension (Gaviscon Advance) in the management of laryngopharyngeal reflux. *Eur Arch Otorhinolaryngol*. 2009;266:243-251.
27. Samuels TL, Johnston N. Pepsin in gastroesophageal and extraesophageal reflux: molecular pathophysiology and diagnostic utility. *Curr Opin Otolaryngol Head Neck Surg*. 2020;28:401-409.
28. Bardhan KD, Strugala V, Dettmar PW. Reflux revisited: advancing the role of pepsin. *Int J Otolaryngol*. 2012;2012:646901.
29. Calvo-Henriquez C, Ruano-Ravina A, Vaamonde P, Martinez-Capoccioni G, Martin-Martin C. Is pepsin a reliable marker of laryngopharyngeal reflux? A systematic review. *Otolaryngol Head Neck Surg*. 2017;157:385-391.
30. Johnston N, Dettmar PW, Ondrey FG, Nanchal R, Lee SH, Bock JM. Pepsin: biomarker, mediator, and therapeutic target for reflux and aspiration. *Ann NY Acad Sci*. 2018;1434:282-289.
31. Weitzendorfer M, Antoniou SA, Schredl P, et al. Pepsin and oropharyngeal pH monitoring to diagnose patients with laryngopharyngeal reflux. *Laryngoscope*. 2019;130:1780-1786.
32. Sereg-Bahar M, Jerin A, Hocevar-Boltezar I. Higher levels of total pepsin and bile acids in the saliva as a possible risk factor for early laryngeal cancer. *Radiol Oncol*. 2015;49:59-64.
33. Dai YF, Tan JJ, Deng CQ, Liu X, Lv ZH, Li XP. Association of pepsin and DNA damage in laryngopharyngeal reflux-related vocal fold polyps. *Am J Otolaryngol*. 2020;41:102681.
34. Johnston N, Yan JC, Hoekzema CR, et al. Pepsin promotes proliferation of laryngeal and pharyngeal epithelial cells. *Laryngoscope*. 2012;122:1317-1325.
35. Li H, Zhang S, Zhou S, et al. Pepsin enhances glycolysis to promote malignant transformation of vocal fold leukoplakia epithelial cells with dysplasia. *Eur Arch Otorhinolaryngol*. 2022;280:1841-1854.
36. Niu K, Guo C, Teng S, et al. Pepsin promotes laryngopharyngeal neoplasia by modulating signaling pathways to induce cell proliferation. *PLoS One*. 2020;15:e0227408.
37. Tan JJ, Wang L, Mo TT, Wang J, Wang MG, Li XP. Pepsin promotes IL-8 signaling-induced epithelial-mesenchymal transition in laryngeal carcinoma. *Cancer Cell Int*. 2019;19:64.
38. Doukas PG, Vageli DP, Sasaki CT, Judson BL. Pepsin promotes activation of epidermal growth factor receptor and downstream oncogenic pathways, at slightly acidic and neutral pH, in exposed hypopharyngeal cells. *Int J Mol Sci*. 2021;22:4275.
39. Adams J, Heintz P, Gross N, et al. Acid/pepsin promotion of carcinogenesis in the hamster cheek pouch. *Arch Otolaryngol Head Neck Surg*. 2000;126:405-409.

40. Kelly EA, Samuels TL, Johnston N. Chronic pepsin exposure promotes anchorage-independent growth and migration of a hypopharyngeal squamous cell line. *Otolaryngol Head Neck Surg*. 2014;150:618-624.
41. Samuels TL, Zimmermann MT, Zeighami A, et al. RNA sequencing reveals cancer-associated changes in laryngeal cells exposed to non-acid pepsin. *Laryngoscope*. 2021;131:121-129.
42. Johnston N, Dettmar PW, Bishwokarma B, Lively MO, Koufman JA. Activity/stability of human pepsin: implications for reflux attributed laryngeal disease. *Laryngoscope*. 2007;117:1036-1039.
43. Samuels TL, Yan K, Patel N, et al. Alginates for protection against pepsin-acid induced aerodigestive epithelial barrier disruption. *Laryngoscope*. 2022;132:2327-2334.
44. Hirschowitz B. Pepsin in the pathogenesis of peptic ulceration. In: Halter F, Garner A, Tytgat GNJ, eds. *Mechanisms of Peptic Ulcer Healing*. Kluwer Academic Publishers; 1990:183-194.
45. Tobey NA, Hosseini SS, Caymaz-Bor C, Wyatt HR, Orlando GS, Orlando RC. The role of pepsin in acid injury to esophageal epithelium. *Am J Gastroenterol*. 2001;96:3062-3070.
46. Caviglia R, Ribolsi M, Maggiano N, et al. Dilated intercellular spaces of esophageal epithelium in nonerosive reflux disease patients with physiological esophageal acid exposure. *Am J Gastroenterol*. 2005;100:543-548.
47. Weijenborg PW, Smout AJ, Verseijden C, et al. Hypersensitivity to acid is associated with impaired esophageal mucosal integrity in patients with gastroesophageal reflux disease with and without esophagitis. *Am J Physiol Gastrointest Liver Physiol*. 2014;307:G323-G329.
48. Reichel O, Mayr D, Durst F, Berghaus A. E-cadherin but not beta-catenin expression is decreased in laryngeal biopsies from patients with laryngopharyngeal reflux. *Eur Arch Otorhinolaryngol*. 2008;265:937-942.
49. Im NR, Kim B, Jung KY, Baek SK. Matrix metalloproteinase-7 induces E-cadherin cleavage in acid-exposed primary human pharyngeal epithelial cells via the ROS/ERK/c-Jun pathway. *J Mol Med*. 2022;100:313-322.
50. Im NR, Lee DY, Kim B, et al. Role of matrix metalloproteinases 7 in the pathogenesis of laryngopharyngeal reflux: decreased E-cadherin in acid exposed primary human pharyngeal epithelial cells. *Int J Mol Sci*. 2019;20:5276.
51. Jovov B, Que J, Tobey NA, Djukic Z, Hogan BL, Orlando RC. Role of E-cadherin in the pathogenesis of gastroesophageal reflux disease. *Am J Gastroenterol*. 2011;106:1039-1047.
52. Nawrocki-Raby B, Gilles C, Polette M, et al. Upregulation of MMPs by soluble E-cadherin in human lung tumor cells. *Int J Cancer*. 2003;105:790-795.
53. David JM, Rajasekaran AK. Dishonorable discharge: the oncogenic roles of cleaved E-cadherin fragments. *Cancer Res*. 2012;72:2917-2923.
54. Johnston N, Samuels TL, Goetz CJ, et al. Oral and inhaled fosamprenavir reverses pepsin-induced damage in a laryngopharyngeal reflux mouse model. *Laryngoscope*. 2022;133:S1-S11.
55. Maretzky T, Reiss K, Ludwig A, et al. ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. *Proc Natl Acad Sci U S A*. 2005;102:9182-9187.
56. Grobelny D, Poncz L, Galardy RE. Inhibition of human skin fibroblast collagenase, thermolysin, and Pseudomonas aeruginosa elastase by peptide hydroxamic acids. *Biochemistry*. 1992;31:7152-7154.
57. Ma D, Wu W, Yang G, Li J, Li J, Ye Q. Tetrahydroisoquinoline based sulfonamide hydroxamates as potent matrix metalloproteinase inhibitors. *Bioorg Med Chem Lett*. 2004;14:47-50.
58. Uttamchandani M, Wang J, Li J, et al. Inhibitor fingerprinting of matrix metalloproteases using a combinatorial peptide hydroxamate library. *J Am Chem Soc*. 2007;129:7848-7858.
59. Jaccard N, Griffin LD, Keser A, et al. Automated method for the rapid and precise estimation of adherent cell culture characteristics from phase contrast microscopy images. *Biotechnol Bioeng*. 2014;111:504-517.
60. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9:676-682.
61. Lee SH, Samuels T, Bock JM, Blumin JH, Johnston N. Establishment of an immortalized laryngeal posterior commissure cell line as a tool for reflux research. *Laryngoscope*. 2015;125:E73-E77.
62. Goldberg HI, Dodds WJ, Gee S, Montgomery C, Zboralske FF. Role of acid and pepsin in acute experimental esophagitis. *Gastroenterology*. 1969;56:223-230.
63. Johnson LF, Harmon JW. Experimental esophagitis in a rabbit model. Clinical relevance. *J Clin Gastroenterol*. 1986;8(suppl 1):26-44.
64. Nagahama K, Yamato M, Nishio H, Takeuchi K. Essential role of pepsin in pathogenesis of acid reflux esophagitis in rats. *Dig Dis Sci*. 2006;51:303-309.
65. Lal M, Caplan M. Regulated intramembrane proteolysis: signaling pathways and biological functions. *Physiology (Bethesda)*. 2011;26:34-44.
66. Hu QP, Kuang JY, Yang QK, Bian XW, Yu SC. Beyond a tumor suppressor: soluble E-cadherin promotes the progression of cancer. *Int J Cancer*. 2016;138:2804-2812.
67. Charalabopoulos A, Golias C. E-cadherin expression in Barrett's esophagus and esophageal carcinoma. *Esophagus*. 2014;11:153-161.
68. Samuels TL, Altman KW, Gould JC, et al. Esophageal pepsin and proton pump synthesis in Barrett's esophagus and esophageal adenocarcinoma. *Laryngoscope*. 2019;129:2687-2695.
69. Gill GA, Johnston N, Buda A, et al. Laryngeal epithelial defenses against laryngopharyngeal reflux: investigations of E-cadherin, carbonic anhydrase isoenzyme III, and pepsin. *Ann Otol Rhinol Laryngol*. 2005;114:913-921.
70. Yuksel H, Ocalan M, Yilmaz O. E-cadherin: an important functional molecule at respiratory barrier between defence and dysfunction. *Front Physiol*. 2021;12:720227.
71. Kim B, Lee HJ, Im NR, et al. Effect of matrix metalloproteinase inhibitor on disrupted E-cadherin after acid exposure in the human nasal epithelium. *Laryngoscope*. 2018;128:E1-e7.
72. Ferber EC, Kajita M, Wadlow A, et al. A role for the cleaved cytoplasmic domain of E-cadherin in the nucleus. *J Biol Chem*. 2008;283:12691-12700.
73. Choi YS, Na HG, Bae CH, Song SY, Kim YD. Pepsin exposure in a non-acidic environment upregulates mucin 5AC (MUC5AC) expression via matrix metalloproteinase 9 (MMP9)/nuclear factor κB (NF-κB) in human airway epithelial cells. *Int Forum Allergy Rhinol*. 2021;11:894-901.
74. Aldhahrani A, Powell J, Ladak S, et al. The potential role of bile acids in acquired laryngotracheal stenosis. *Laryngoscope*. 2018;128:2029-2033.
75. Montiel-Jarquín ÁJ, Lara-Cisneros LGV, López-Colombo A, Solís-Mendoza HA, Palmer-Márquez ML, Romero-Figueroa MS. Expression of metalloproteinase-9 in patients with mild and severe forms of gastroesophageal reflux disease. *Cir Cir*. 2019;87:436-442.
76. Davelaar AL, Straub D, Buttar NS, Fockens P, Krishnadath KK. Active matrix metalloproteases are expressed early on and are high during the Barrett's esophagus malignancy sequence. *Scand J Gastroenterol*. 2015;50:321-332.
77. Grzelczyk WL, Wróbel-Roztropiński A, Szemraj J, et al. Matrix metalloproteinases (MMPs) and inhibitor of metalloproteinases (TIMP) mRNA and protein expression in laryngeal squamous cell carcinoma. *Arch Med Sci*. 2019;15:784-791.
78. Grimm M, Lazariotou M, Kircher S, et al. MMP-1 is a (pre-)invasive factor in Barrett-associated esophageal adenocarcinomas and is associated with positive lymph node status. *J Transl Med*. 2010;8:99.

79. Cheung WY, Zhai R, Bradbury P, et al. Single nucleotide polymorphisms in the matrix metalloproteinase gene family and the frequency and duration of gastroesophageal reflux disease influence the risk of esophageal adenocarcinoma. *Int J Cancer*. 2012;131:2478-2486.
80. Niland S, Riscanevo AX, Eble JA. Matrix metalloproteinases shape the tumor microenvironment in cancer progression. *Int J Mol Sci*. 2021;23:146.
81. Blaine-Sauer S, Samuels TL, Yan K, Johnston N. The protease inhibitor amprenavir protects against pepsin-induced esophageal epithelial barrier disruption and cancer-associated changes. *Int J Mol Sci*. 2023;24:6765.
82. Hvid-Jensen F, Pedersen L, Funch-Jensen P, Drewes AM. Proton pump inhibitor use may not prevent high-grade dysplasia and oesophageal adenocarcinoma in Barrett's oesophagus: a nationwide study of 9883 patients. *Aliment Pharmacol Ther*. 2014;39:984-991.
83. GlaskoSmithKline. Lexiva prescribing information. 2023. Accessed March 5, 2023 https://gskpro.com/content/dam/global/hcpportal/en_US/Prescribing_Information/Lexiva/pdf/LEXIVA-PI-PIL.PDF
84. Dasgupta A, Schlette E. Rapid in vitro conversion of fosphenytoin into phenytoin in sera of patients with liver disease: role of alkaline phosphatase. *J Clin Lab Anal*. 2001;15:244-250.
85. Bourne GH. Alkaline phosphatase in taste buds and nasal mucosa. *Nature*. 1948;161:445-446.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Samuels TL, Blaine-Sauer S, Yan K, Johnston N. Amprenavir inhibits pepsin-mediated laryngeal epithelial disruption and E-cadherin cleavage in vitro. *Laryngoscope Investigative Otolaryngology*. 2023;8(4):953-962. doi:10.1002/lio2.1102