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Preclinical stability assessment of EPICERTIN, a biotherapeutic candidate for ulcerative colitis mucosal healing.

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PRECLINICAL STABILITY ASSESSMENT OF EPICERTIN, A BIOTHERAPEUTIC CANDIDATE FOR ULCERATIVE COLITIS MUCOSAL HEALING

By

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B.S., Western Kentucky University, 2021

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DEDICATION

This thesis is dedicated to my late father,

Robert Cecil Sr.

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I would like to thank my advisor, Dr. Nobuyuki Matoba, for fostering a supportive environment where curiosity and growth thrive. Thank you to the members of the Matoba Lab for the shared laughter, memes, crosswords, and music bingo nights. I am grateful to the Department of Pharmacology and Toxicology and my committee members for their contributions to my development as a scientist. Importantly, thank you to my husband, Kyle Kittle, and my family as their support has made this work possible.

ABSTRACT

PRECLINICAL STABILITY ASSESSMENT OF EPICERTIN, A BIOTHERAPEUTIC CANDIDATE FOR ULCERATIVE COLITIS MUCOSAL HEALING

Wendy Michelle Kittle

January 18, 2024

EPICERTIN is a biotherapeutic candidate for mucosal healing in ulcerative colitis (UC), a major type of inflammatory bowel disease (IBD). Despite the critical role of mucosal healing in achieving successful remission of UC, there are currently no targeted mucosal healing agents available. EPICERTIN has previously demonstrated epithelial repair and mucosal healing activity in colon epithelial cells, murine colitis models, and tissue explants from IBD patients, highlighting its therapeutic potential to meet this unmet need for UC patients. The development of appropriate chemistry, manufacturing, and controls (CMC) data will facilitate our ultimate goal to bring EPICERTIN to the clinic for a first-in-human (FIH) clinical study. To this end, this work describes the regulation-compliant long-term and forced degradation stability assessments of EPICERTIN DS to generate molecular stability and physicochemical property profiles.

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CHAPTER I: INTRODUCTION

1.1. Ulcerative colitis (UC) epidemiology and unmet treatment need

Inflammatory bowel disease (IBD) is an umbrella term for chronic, relapsing inflammatory conditions affecting the gastrointestinal (GI) tract. The two main forms of IBD are Crohn's disease (CD) characterized by discontinuous, transmural inflammation throughout the GI tract, and ulcerative colitis (UC) which primarily impacts the innermost lining of the distal GI tract, usually beginning at the rectum and extending into the colon [1]. IBD affects approximately 1.6 million people (780,000 CD and 907,000 UC), with 70,000 new cases diagnosed each year in the United States and has an estimated annual financial burden of $14.6 billion to $31.6 billion [2]. UC causes a range of symptoms that severely affect quality of life such as abdominal pain, diarrhea, and urgency, among others [1, 3]. Additionally, patients with long-standing IBD, particularly UC, have a significantly increased risk of developing colitis-associated cancer [4-6]. While there is no cure for IBD, current therapies aim to manage inflammation, control symptoms, and improve quality of life.

The etiology of UC is elusive and is likely caused by a combination of genetic, environmental, and immune system factors [1, 7-9], therefore, treatment for UC typically involves a step-up approach, tailored to disease severity and response to previous therapies, with the goal of achieving remission with minimal side effects and complications [10, 11]. Aminosalicylates (5-ASAs), are the first-line medications for

mild-to-moderate UC, which can be targeted locally in the gut to reduce inflammation and are generally well-tolerated with potential innocuous side effects caused by dose-related intolerance and rarely by allergy [11-18]. Corticosteroids are acutely used for moderate-to-severe UC or if 5-ASAs fail. These anti-inflammatory drugs globally activate glucocorticoid-responsive elements that influence multiple signal transduction pathways to alleviate inflammation, but prolonged use can lead to predictable side effects like weight gain, insomnia, osteoporosis, and increased risk of infection [18-20]. Immunomodulators, also known as immunosuppressants, are nonbiological agents that directly target the immune system's cellular processes and are used when the 5-ASAs or corticosteroid treatment is inadequate. These agents suppress the overactive immune response through varied mechanisms and largely lack robust clinical efficacy data [21, 22]. Toxicity with immunomodulator use is not uncommon, with risk of side effects like infection, and bone marrow toxicity, and nephrotoxicity [10, 11, 13, 23-26], which requires this class of agents to be frequently monitored for safety. Tofacitinib, a JAK inhibitor approved in 2018, targets multiple immune cytokine pathways involved in UC pathogenesis by acting on the signal transducers and activators of transcription (STAT) pathway [27]. While effective, its use carries the risks of increased immunosuppression. Biologics (e.g., anti-TNF agents), are reserved for severe UC or patients who have not responded to other therapies. Ustekinumab is an interleukin 12/23 monoclonal antibody inhibitor, approved in 2019, that demonstrates efficacy in inducing and maintaining clinical remission in UC [28]. Post-market side effects remain under observation. These targeted medications offer high rates of efficacy but also a higher price tag and potential for serious opportunistic infections and loss of efficacy as a result of anti-drug antibodies

[18, 23, 29-35]. For the most severe medication-resistant cases, surgical resection of the affected colon may be required, which is a major procedure with potential complications and can greatly impact the patient's quality of life [10, 11].

Similar to UC, treatment for CD lacks a one-size-fits-all approach. A multi-pronged strategy is typically employed, combining medications, dietary modifications, and potential surgery for severe cases. Despite widespread use, 5-ASAs offer little benefit in reducing CD complications or surgical rates [36-38]. However, some benefit over placebo might exist for preventing postoperative CD following ileal resection [39, 40]. The established role of the gut microbiome in promoting intestinal inflammation has led to exploration of manipulating bowel flora with antibiotics and probiotics, with antibiotics demonstrating success in limiting anaerobic bacterial overgrowth and inflammation in the intestinal mucosa and little to no evidence of the efficacy of probiotics [40, 41]. Certain corticosteroids (e.g., prednisone and prednisolone) manage symptoms for moderate to severe CD but do not promote mucosal healing, and their significant toxicities and lack of long-term efficacy and make corticosteroids unsuitable for remission maintenance [42, 43]. Immunomodulators, including thiopurines and methotrexate, can be used as standalone therapy or adjunctive therapy with anti-TNF drugs. Thiopurines inhibit purine synthesis and reduce cell-mediated immunity by suppressing T cells and natural killer cells. However, they are associated with side effects ranging from flu-like symptoms to more serious adverse events like leukopenia, pancreatitis, liver damage, lymphoma, serious infections, and nonmelanoma skin cancer [41, 44, 45]. Methotrexate also inhibits purine synthesis through folate antagonism, although its specific cell targets remain unclear. It can cause nausea, headaches, and

fatigue, alongside potentially serious adverse events like liver damage with fibrosis, bone marrow suppression, infections, and rarely, hypersensitivity pneumonitis [46]. Due to safety concerns, immunomodulator treatment necessitates routine monitoring. Calcineurin inhibitors are used to treat severe, steroid-refractory CD, but their use is limited due to the availability of safer and more effective therapies [47]. Biologic therapy with anti-TNF agents revolutionized CD treatment, as these drugs are effective for inducing and maintaining remission while promoting mucosal healing [41], although they share similar side effects and limitations as those discussed for UC therapy. Finally, surgery is a potential option for 50-70% of CD patients within 5-20 years of diagnosis [48, 49]. While it achieves rapid remission for medically refractory disease, surgery is not curative and carries a high risk of future recurrence [48].

Beyond symptom relief, the current clinical goals for IBD extend to achieving deeper levels of healing the mucosa, with the potential to predict and maintain remission [50-52] as well as reduce the need for surgery and improve quality of life. Mucosal healing refers to the restoration of the colonic mucosa's epithelial barrier to a healthy, homeostatic state, and is often assessed via endoscopy [53-56], as the restoration of the barrier ameliorates the propagation of the disease by protection of the underlying tissue from gut bacteria. Finer levels of analysis on colon tissue biopsies can confirm histological remission, the absence of microscopic inflammation in [57-59]. Emerging tools like confocal laser endomicroscopy (CLE) offer even greater resolution and real-time visualization of mucosal healing and barrier integrity at the cellular level [60-63]. Epithelial repair is a critical step in mucosal healing as recovering the intestinal epithelial barrier inhibits inflammation caused by entry of bacteria into the mucosa. Thus, an agent

that can restore the epithelial barrier and facilitate mucosal healing without suppressing immune function may fill a current treatment gap for IBD.

1.2. EPICERTIN's potential for UC: efficacy and epithelial repair

EPICERTIN is a variant of the cholera toxin B subunit (CTB). Cholera toxin is a major virulence factor of *Vibrio cholerae* composed of a catalytic A subunit (CTA) and the non-toxic CTB [64, 65]. CTB is the homopentameric component and mediates toxin entry into intestinal epithelial cells via binding to the cell-surface GM1 ganglioside receptor and subsequent retrograde transport to the endoplasmic reticulum (ER) [64, 65]. There, CTA dissociates from CTB and induces uncontrolled water and ion secretion in the colon [64, 65]. EPICERTIN was originally engineered for cholera vaccine production in the *Nicotiana benthamiana* plant platform, aimed at overcoming cost and scale hurdles in CTB biomanufacturing [66, 67]. EPICERTIN is a CTB variant possessing a C-terminal hexapeptide ER-retention motif containing -KDEL, facilitating its production in *N. benthamiana*. While the modification did not affect the GM1-binding affinity, molecular stability, or oral immunogenicity of the original molecule [67], EPICERTIN serendipitously exhibited potential as a novel therapeutic for UC. EPICERTIN's mucosal healing activity has been further characterized, reveling insights about its epithelial repair mechanisms and efficacy. Oral administration of EPICERTIN, but not CTB, facilitates epithelial repair and mucosal healing in dextran sulfate sodium (DSS)-induced acute and chronic colitis mouse models [68, 69]. EPICERTIN's unique mucosal healing activity is attributed to the molecule's capacity to interact with the KDEL receptor and subsequently activate the inositol-requiring enzyme 1/X-box binding protein 1 arm of an unfolded protein response in colon epithelial cells. Additionally, EPICERTIN can be administered

topically to the colon or by oral gavage to alleviate DSS-induced colitis in mice [70]. While oral administration of EPICERTIN requires prerequisite neutralization of stomach acid, we previously addressed this limitation by developing a prototype enteric-coated oral formulation for pH-dependent release of EPICERTIN in the colon [71]. EPICERTIN also had a significant impact on specific immune cell populations in the colon in naive mice [68], which suggests that EPICERTIN may have the potential to modulate the immune cell profile in the colon to promote mucosal healing.

Further evidence for EPICERTIN's effectiveness came from studies on human colon tissues obtained from IBD patients undergoing total colectomy [72] in which *ex vivo* assays showed EPICERTIN induced wound healing responses as indicated by the presence of viable, proliferating Ki67 positive crypts, whereas these effects were not observed with CTB or PBS treatments. Notably, EPICERTIN treatment significantly increased the expression of multiple genes associated with wound healing (including TGFB1, CDH1, and WNT5A) in colectomy tissues from various IBD patients. The most pronounced effect was observed in tissue from a 20-year-old male UC patient, where EPICERTIN upregulated the expression of 79 out of 84 wound healing-related genes. Overall, these summarized findings highlight EPICERTIN's potential as a targeted mucosal healing agent for UC therapy.

1.3. Advancing EPICERTIN's preclinical development through regulation-compliant stability testing

EPICERTIN's current developmental stage involves the generation of appropriate chemistry, manufacturing and controls (CMC), following regulatory guidance from the Food and Drug Administration (FDA) and the International Council for Harmonisation

(ICH). CMC encompasses the scientific understanding, large-scale production, and quality control procedures that ensure the safety and efficacy of a drug product. This involves defining the drug's structure, developing a controlled manufacturing process, and implementing robust analytical methods to consistently produce a high-quality drug substance and drug product. Stability testing is a crucial task at this stage of development. According to ICH Q1A(R2) guidance, the purpose of stability testing a drug is twofold: to demonstrate how the quality of the drug is affected upon exposure to a variety of environmental factors and to establish a retest period with recommended storage and shelf-life conditions [73]. Stability of a drug is assessed by a battery of tests evaluating its physicochemical, functional, and basic attributes such as identity, purity, potency, and safety, all of which inform specifications that the drug must meet for future stages of development [74]. Stability testing is not performed using a “one size fits all” approach; the intended shelf-life and storage conditions of a specific drug determine what stability conditions must be tested.

If long-term studies are conducted, a minimum of 12 months of stability data, with timepoints at three-month intervals, are required at the time of investigational new drug (IND) application submission [73]. The storage conditions of the drug should be tested for stability, at minimum, at 25°C at 60% relative humidity (RH). If the drug is intended for storage in a refrigerator, stability at 5°C should also be assessed [73]. If a drug is found to be exceptionally stable at 12 months, stress testing may be utilized to identify likely degradation pathways and validate the stability indicating power of the analytical procedures used [73].

By unveiling a drug's susceptibility to potential stressors, forced degradation studies significantly enhance the understanding of a drug's molecular properties [75, 76]. This information provides crucial information necessary for securing regulatory approval. This rigorous testing exposes potential degradation pathways impacting purity and potency, guiding formulation and packaging optimization, and predicting long-term stability under normal conditions [75, 76]. Ultimately, this safeguards patient safety while informing the development of reliable analytical methods [75, 76].

During initial contact with the FDA through PreIND communication, expectations regarding the CMC information for EPICERTIN development were outlined. A thorough characterization of EPICERTIN was recommended as development progresses. This characterization should encompass critical quality attributes, mechanism(s) of action, potential degradants under various conditions, and their impact on product quality. This information would be used to guide future characterization needs for later-phase development. For the initial Phase I safety and tolerability study, a 95% purity specification was deemed acceptable. However, a comprehensive evaluation of product purity and impurities was suggested to refine this criterion, acknowledging the potential influence of the analytical method used. Additionally, the establishment of nicotine content was recommended, given its potential presence as a secondary metabolite from the host plant, *N. benthamiana*. The overall focus of the communication emphasized the importance of characterizing EPICERTIN. This characterization would establish a knowledge base to support potential adjustments to specifications and test methods as development progresses towards later-stage clinical trials, which is in accordance with

the ICH Q6b that also provides guidance information about specifications, test procedures, and acceptance criteria for biotechnological/biological products.

The goal of this project is to develop EPICERTIN as a novel mucosal healing biotherapeutic for UC treatment. This thesis describes the evaluation of the stability of EPICERTIN drug substance (DS) (i.e., EPICERTIN at 1 mg/mL in PBS (VWR; VWRl0119-500) at 5 °C and 25°C/60% RH over a long-term 2-year period as well as under forced degradation conditions, which yielded key CMC information to facilitate a future FIH clinical trial.

CHAPTER II: MATERIALS AND METHODS

EPICERTIN biochemical characterization toolbox. The "toolbox" for EPICERTIN DS stability assessments consists of test methods to evaluate test parameters such as purity (SDS-PAGE and size exclusion high performance liquid chromatography (SEC-HPLC)), identity (electro-spray ionization mass spectrometry (ESI-MS) and western blot (WB)), and potency (GM1-capture KDEL-detection (GM1/KDEL) ELISA). To assess stability, specifications and acceptance criteria for each test method were established and justified based on multiple EPICERTIN DS batch measurements at release as advised by ICH guidelines (Q6A) and Pre-IND communication with the FDA. The detailed methods for EPICERTIN's test methods and specifications are described below and can also be referenced in brevity in Table 1.

SDS-PAGE. Reducing SDS-PAGE was performed to evaluate purity, using a pre-cast Mini-PROTEAN TGX 4-20% gradient gel (Bio-Rad). EPICERTIN DS samples were diluted into 2X sample buffer containing 4% SDS and 10% BME and incubated at 95 °C for 10 minutes. Samples were loaded (10 µg), and the gel was run at 200V for 35 min, Coomassie (R-250) stained for 1 hour, then destained, rocking in destain buffer (12% ethanol, 12% acetic acid) for 5 hours. Colorimetric images were captured by Amersham Imager 600. Evaluation of monomeric content by densitometry assessment was performed using GelAnalyzer software (Version 19.1), with a specification of $\geq$ 95% monomer. To be discussed in the following paragraph, Pre-IND communication with the

FDA defined a purity threshold for pentameric EPICERTIN as ≥ 95% pure by SEC-HPLC assessment. As such, we held the same standard for the purity threshold for monomeric EPICERTIN as ≥ 95% pure by SDS-PAGE assessment.

SEC-HPLC. Percent of intact pentamer EPICERTIN (61.4 kDa) was assessed as described previously [71] to evaluate purity. Briefly, samples at a concentration of 1 mg/mL were run over a Tosoh TSKgel SuperSW300 column using PBS (VWR; VWRL0119-0500) running buffer. Evaluation of chromatograms considers retention time in minutes and area under the curve (AUC, in milli absorbance units (mAu)), with specifications of retention time (16.4+0.2 min) and ≥95% AUC. The AUC specification was informed by Pre-IND communication with the FDA.The retention time was redefined based on multiple batch measurement validation after column calibration.

ESI-MS. Samples obtained from freshly opened vials were sent to The Scripps Research Institute (San Diego, CA) for analysis. This intact mass analysis is an EPICERTIN identity method, used to identify the presence and content of C-terminally intact and truncated species as described previously [77]. Intact monomeric mass is assessed (12,280 Da), with a specification of 12,280 ±3 Da. This specification indicates the theoretical intact mass of an EPICERTIN monomer (12280 Da) as well as a 3 Da range for measurement tolerance that accounts for this acceptable deviation in resolving power of the mass analyzer.

WB. This test method evaluates identity and was implemented in this work a single time to demonstrate the identity of EPICERTIN dimer, as seen in Figure 2. SDS-PAGE gels were run according to the procedure described above, with 500 ng protein per sample loaded. Transblot turbo (BioRad) was used for semi-dry transfer to PVDF

membrane (BioRad). The membrane was blocked using PBST (1× PBS, 0.05% Tween 20) containing 5% non-fat dry milk (PBSTM), rocking at 4°C overnight. The membrane was washed 3 times with PBST, 5 min each, rocking. The membrane was stained with primary antibody (9F9C7 Rat anti-CTB mAb [78]) diluted 1:20,000 in 1% PBSTM for 1 hour at room temperature, rocking. The membrane was washed again, and then stained with secondary antibody (Goat anti Rat IgG -HRP; Southern Biotech 3030-05) diluted 1:2,000 in 1% PBSTM for 1 hour at room temperature, rocking. The membrane was washed again, and chemiluminescent detection reagent (Amersham ECL Prime) was added to the membrane according to manufacturer's instructions. Chemiluminescent images were captured by Amersham Imager 600.

GM1/KDEL ELISA. Potency was assessed by a GM1-binding KDEL-detection ELISA as previously described [71, 77]. Briefly, plates were coated with 2 μg/mL GM1 ganglioside (Sigma) for 2 hours at room temperature (20–23°C). The plate was washed three times with PBST and blocked with 5% PBSTM overnight at 4°C. Samples were added at a top concentration of 2 μg/mL in 1% PBSTM, diluted 1:2 across the plate, and then incubated for 2 hours at room temperature. Primary antibody (10C3 mouse anti-KDEL monoclonal antibody (1:1000; Abcam) was applied and incubated at room temperature for 1 hour. Secondary antibody (goat anti-mouse IgG-HRP (1:5000; SouthernBiotech 1030-05)), was applied and incubated at room temperature for 1 hour. TMB (BioFX™ TMB One Component HRP Substrate, Surmodics) was used to detect GM1 binding. Absorbance was measured at 450 nm in a Synergy HT plate reader (BioTek) after the reaction was stopped. Evaluation of GM1/KDEL ELISA considers the specification of $\%EC_{50}$ shift $< \pm$ 20% reference standard. This acceptance criterion is

generally considered a stringent window for developmental phases prior to Phase III. The traditional window at an early stage is often wider and is then narrowed down to < ± 20% at later developmental stages to support efficacy and assure lot-to-lot drug preparations are biologically active and consistently manufactured [79, 80], for which this < ± 20% acceptance criteria is recommended upon assay validation [81].

Production of EPICERTIN. EPICERTIN DS was produced in *Nicotiana benthamiana* plants and purified to > 95% homogeneity with an endotoxin level of < 1 endotoxin units per mg, as described previously [77] but with modification in the final chromatography step to selectively purify the proteins with fully intact C-terminus. EPICERTIN DS was packaged into 5 mL polyethylene terephthalate glycol (PETG) vials with a 2.5 mL fill and concentration of 1.06 mg/mL in PBS. Purity (SDS-PAGE and SEC-HPLC), identity (ESI-MS) and potency (GM1/KDEL ELISA) were verified.

Long-term stability assessment experimental design. EPICERTIN DS was produced as described above and stored for 2 years in a monitored refrigerator or an environment-controlled chamber (CARON model CRSY102 environmental chamber) set at either 5 °C or 25 °C/60% RH. At each time point (0, 1, 3, 6, 9, 12, and 24 months), a new vial from both temperatures was pulled, protein concentration was measured by Thermo Scientific™ NanoDrop™ OneC, and the pH of each vial was measured using a VWR SB90M5 Benchtop sympHony Meter. Analytical assessments for identity, purity, and potency were performed as follows.

Long-term stability test methods. At each time point, purity (SDS-PAGE; SEC-HPLC), identity (ESI-MS; WB), and potency (GM1/KDEL ELISA) were assessed as described above. Appearance, concentration, physicochemical properties, and Bioburden

(contracted microbial enumeration test (USP<61>)) were also assessed at each time point. Appearance was evaluated by a specification of clear, colorless liquid, free of visible particles (CCLFVP), and concentration evaluated by a specification of 1 $\pm$ 0.2 mg/mL. Physicochemical properties and Bioburden were evaluated by specifications of pH 7.4 $\pm$ 0.2 and < 10 colony forming units (CFU)/mL, respectively.

Forced degradation stability assessment experimental design. EPICERTIN DS was produced as described above and stored at 5 °C. EPICERTIN DS was subjected to several forced degradation conditions: temperature (40, 50, and 60 °C for 24, 48, and 72 hours), freeze-thaw (1, 2, 3, 4, and 5 cycles), pH (pH 1, 4, 7, and 10), agitation (24 and 48 hours), and oxidation (0.2% and 2% H_2O_2). Temperature: 20 µL of EPICERTIN DS was aliquoted into 2 mL screw cap vials. Vials were placed in water baths set for 40, 50, and 60 °C for 24, 48, and 72 hours. Freeze/thaw: 100 µL of EPICERTIN DS was aliquoted into 2 mL screw cap vials and exposed to up to five cycles of -20°C/25°C. pH: EPICERTIN DS samples were pH-adjusted using either 1 N NaOH or 1 M HCl to reach final pH values of 1, 4, 7, and 10, and 20 µL of each sample was aliquoted into 1.7 mL microcentrifuge tubes and stored at 5°C for 48 hours. Agitation: 100 µL EPICERTIN DS samples were aliquoted into 1.7 mL microcentrifuge tubes and agitated at room temperature by vortexing at maximum speed (Denville Scientific Inc. Vortexer 59A) for 24 and 48 hours. Oxidation: EPICERTIN DS was exposed to a final concentration of 0.2% and 2% hydrogen peroxide (H_2O_2), and 60 µL aliquots were stored in 1.7 microcentrifuge tubes at 5°C for 36 hours. Analytical assessments of purity, potency, and identity were performed as follows.

Forced degradation test methods. For all forced degradation conditions, purity (SEC-HPLC) and potency (GM1/KDEL ELISA) were assessed as described above. In response to SEC-HPLC results, purity was also assessed by SDS-PAGE for all conditions. Oxidation samples were further assessed by ESI-MS intact mass analysis to evaluate oxidation status.

CHAPTER III: RESULTS

3.1. Long-term 2-year stability assessment results

The test methods and the corresponding specifications used to evaluate EPICERTIN DS long-term stability are summarized in Table 1.

Table 1. EPICERTIN stability test parameters, methods, and specifications.

Test Parameter	Test Method	Specification at Release
Appearance	Visible appearance	Clear, colorless liquid, free of visible particles
Identity	ESI-MS	12280 ± 3 Da
Protein Concentration	A_{280}	1 $\pm$ 0.2 mg/mL
Potency	GM1/KDEL ELISA	EC50 shift < ±20% of reference standard
Purity	SEC-HPLC	16.4 $\pm$ 0.2 min retention time and ≥ 95% AUC
Purity	Reducing SDS-PAGE	≥ 95% monomer
Physicochemical Properties	pH	7.4 ± 0.2

The results of appearance, protein concentration, and pH assessments are shown in Table 2. Protein content analysis was carried out by measuring absorbance at 280 nm. The concentration of EPICERTIN DS in PBS, initially prepared at 1 mg/ml and stored at

25 °C/60% RH, was slightly increased beyond specification over 2 years, reaching 1.35 mg/mL at the 24-month time point, whereas the 5 °C counterpart remained within the 1 ± 0.2 mg/mL specification.

Table 2. Long-term assessment of appearance, protein concentration, pH, physicochemical properties, and bioburden.

Test Parameter	**Specification at Release**	**Initial**	**24 months**	
			5 °C	**25 °C/ 60% RH**
Appearance	Clear, colorless liquid, free of visible particles (CCLFVP)	CCLFVP	CCLFVP	CCLFVP
Protein Concentration (mg/mL)	1 ± 0.2	1.05	1.13	1.35
Physicochemical Properties (pH)	7.4 ± 0.2	7.25	7.32	7.25
Safety (CFU/mL)	<10	Pass	Pass	Pass

Identity. ESI-MS intact mass analysis was used to detect degradation of EPICERTIN DS. At the initial timepoint, the intact mass profile showed a major peak at the expected molecular mass for EPICERTIN monomer (~85% 12,280 Da, -KDEL intact) with two minor peaks at the expected molecular masses for C-terminally truncated Leu (~5% 12,038 Da, -KDE intact) and Glu-Leu species (~10% 12,167 Da, -KD intact). As a result of the platform-endogenous carboxy peptidases, these truncated species are consistently present and expected in these proportions in plant made EPICERTIN DS[66, 77]. This method showed that the EPICERTIN DS mass profile remained unchanged at

24 months at both 5°C and 25°C/60% RH and maintained proportions of C-terminally truncated species consistent with the initial time point (Figure 1).

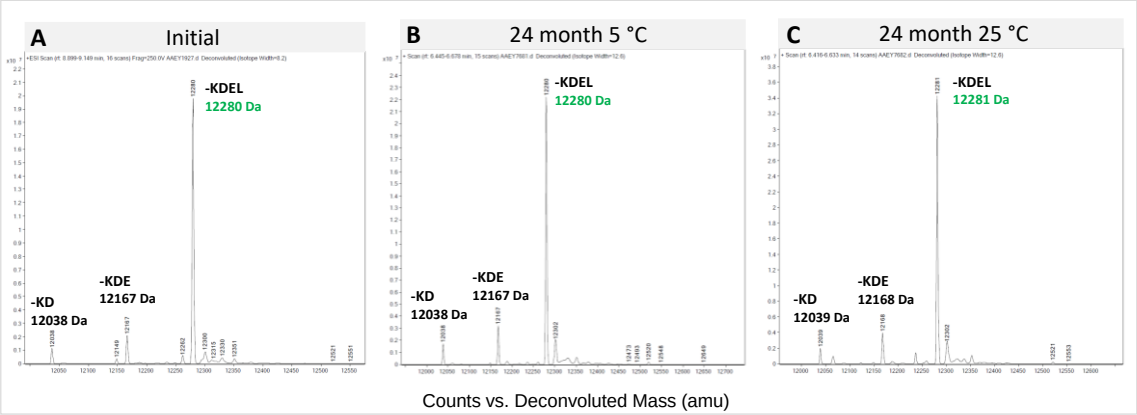

Figure 1. ESI-MS scan (identity) of EPICERTIN DS at 24 months. A) ESI-MS chromatogram demonstrating EPICERTIN DS identity by monomeric intact mass within specification (12280 ± 3 Da), at the initial time point with unchanged truncated content both B) after 24 months at 5 °C, and C) after 24 months at 25 °C/60% RH.

Purity. The purity of EPICERTIN DS was assessed by SDS-PAGE and SEC-HPLC. Reducing SDS-PAGE was used to assess purity of EPICERTIN monomer and demonstrated slight alterations in samples stored at 5 °C (93.8% monomer) or 25 °C/ 60% RH (91.8% monomer) for 24 months (Figure 2). Anti-CTB WB was performed to confirm the identity of EPICERTIN dimer bands around 25 kDa. Purity was also assessed by SEC-HPLC, and the native pentamer conformation of EPICERTIN DS was unaffected upon storage at 5°C for 24 months (Figure 3B), with a retention time of 16.37 min, 100% AUC. Slight degradation of pentamer (16.29 min, 97.5% AUC) after storage at 25°C/ 60% RH for 24 months was observed, as indicated by the presence of a small peak at a higher retention time (20.95 min, 2.5% AUC) than EPICERTIN pentamer (Figure 3C).

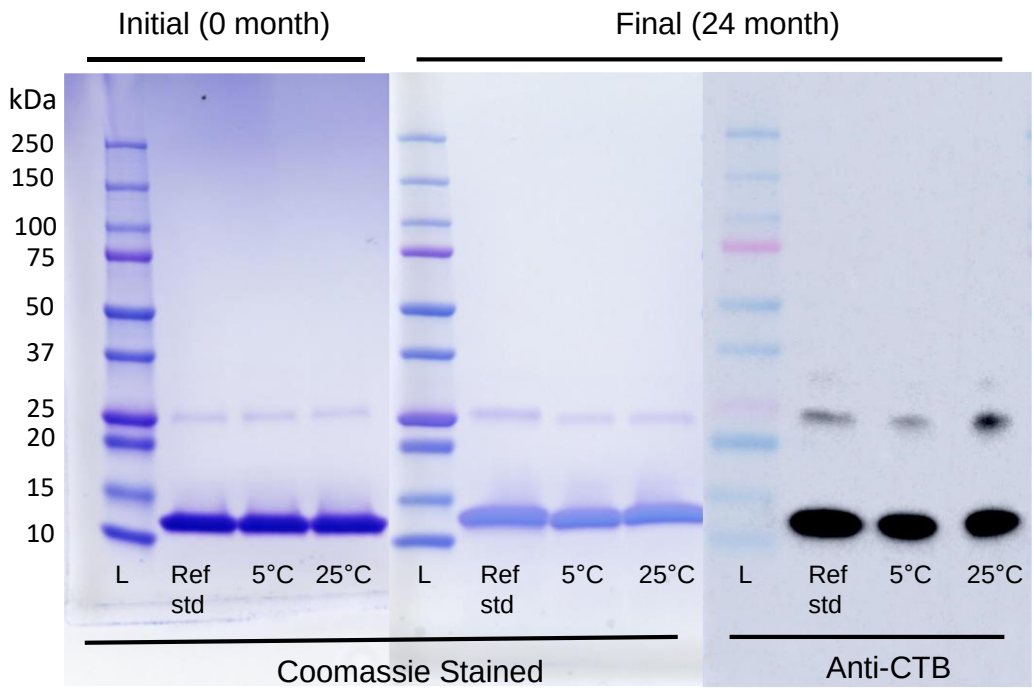

Figure 2. Purity assessment of EPICERTIN DS at 24 months by SDS-PAGE and WB. Coomassie stained SDS-PAGE is shown for initial (0 month) and final (24 month) time points. Anti-CTB WB of SDS-PAGE showing the final timepoint, probed with 9F9C7 mAb [78]. Expected size of denatured EPICERTIN monomer is ~12.3 kDa. Additional bands present are seen at ~25 kDa indicating EPICERTIN dimer.

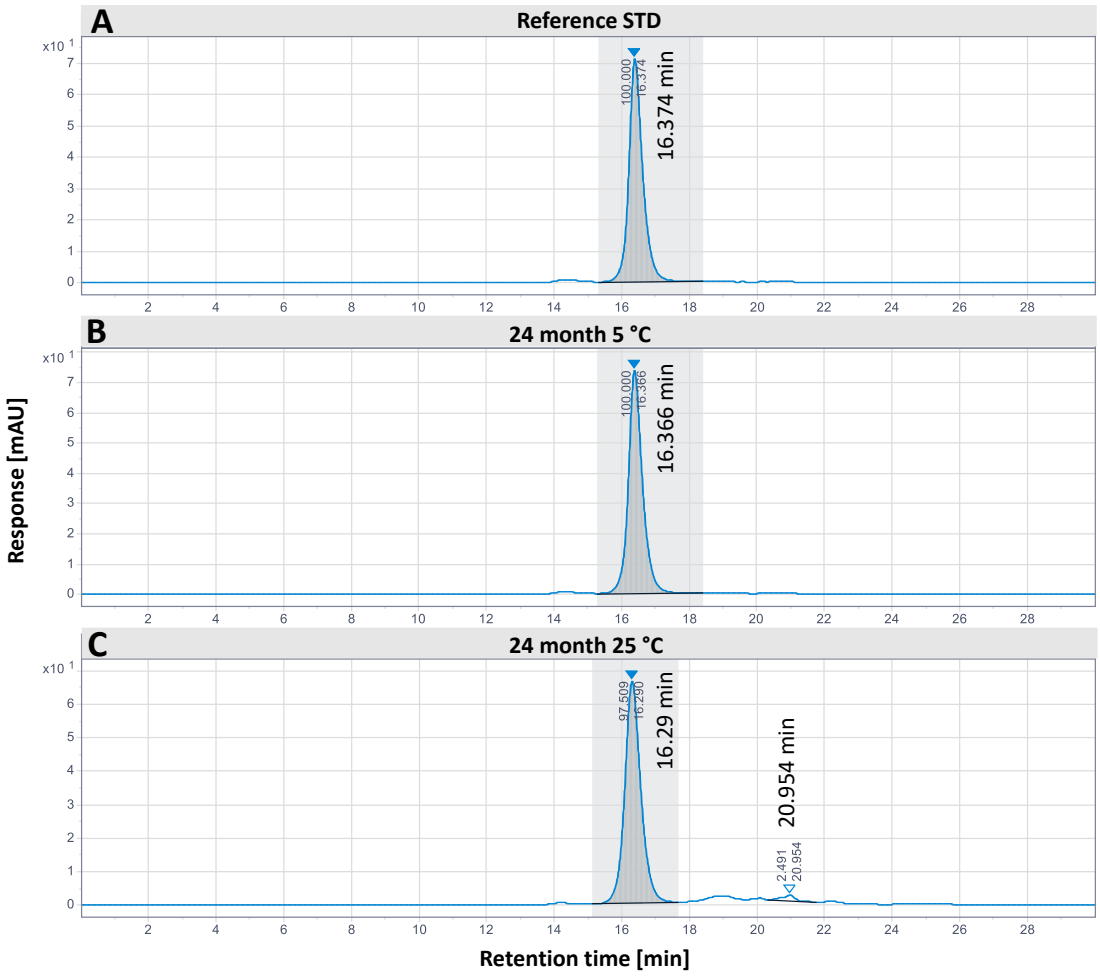

Figure 3. SEC-HPLC (purity) of EPICERTIN DS at 24 months. SEC-HPLC chromatograms demonstrate pentamerized EPICERTIN DS identity within specification (> 95% pentamer) at (B) 5°C, 100% pentamer, and (C) 25°C/60% RH, 97.5% pentamer, at the final time point.

Potency. The GM1/KDEL ELISA, a surrogate biological potency assay [77], was used to gauge the stability of the EPICERTIN DS molecular functions. This assay quantifies GM1-binding activity and confirms the integrity of the C-terminal ER retention motif, both of which are essential for EPICERTIN's mucosal healing activity [70]. Stability samples were assessed using their nominal concentrations (1.06 mg/mL)

for the ELISA. At 24 months of storage, the EC_{50} of EPICERTIN DS was unchanged at 5 °C (< ±20 % EC_{50} shift). However, at 25 °C/ 60% RH, there appeared to be a decrease (%EC_{50} shift of –26.1%) (Figure 4). Since the concentration of EPICERTIN in the 24-month 25 °C/ 60% RH vial had increased (Table 2), we performed another GM1/KDEL ELISA to adjust for the actual concentration. This adjusted analysis confirmed that the EC_{50} of EPICERTIN DS was unchanged after 24 months of storage at 25 °C/ 60% RH (< ± 20 % EC_{50} shift) (data not shown). Overall, the results of the long-term stability assessment suggest that EPICERTIN DS, retains its stability for at least up to 24 months under the tested conditions. A comprehensive summary of results from the long-term stability assessment can be found in Table 3.

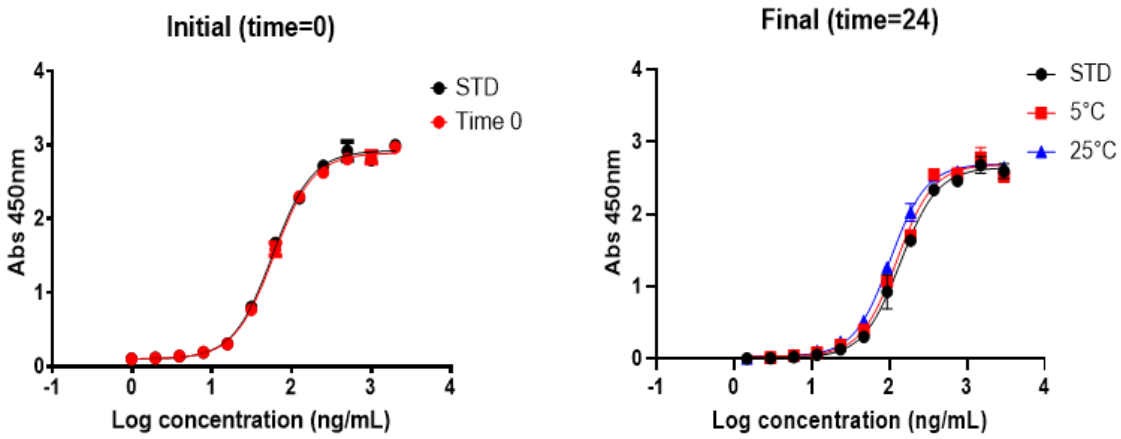

Figure 4. GM1/KDEL ELISA (potency) of EPICERTIN DS at 24 months. (Left) Initial time point potency assessment. (Right) At 24 months, compared with the reference standard (STD), 5 °C is within specification (< ± 20% EC50 shift), whereas 25°C/ 60% RH has an EC50 shift of -26.1%.

Table 3. Results summary of the long-term stability assessment.

					Temperature 5 °C											
Test Parameter	**Test Method**	**Specification**	**0 months**		**1 month**		**3 months**		**6 months**		**9 months**		**12 months**		**24 months**	
Appearance	Visible appearance	Clear, colorless liquid, free of visible particles (CCLFVP)	Pass	CCLFVP	Pass	CCLFVP	Pass	CCLFVP	Pass	CCLFVP	Pass	CCLFVP	Pass	CCLFVP	Pass	CCLFVP
Identity	ESI-MS	12280 ± 3 Da	Pass	12280	Pass	12280	Pass	12281	Pass	12280	Pass	12280	Pass	12280	Pass	12280
Protein Concentration	A_{280}	1 ± 0.2 mg/mL	Pass	1.05	Pass	1.03	Pass	1.06	Pass	1.07	Pass	1.11	Pass	1.13	Pass	1.13
Potency	GM1-KDEL ELISA	EC50 shift < ±20% of reference standard	Pass	+2.85%	Pass	+2.88%	Pass	+16%	Pass	+4.9%	Pass	-7.7%	Fail	-27.2%	Pass	-9.6%
Purity	Size exclusion HPLC	16.4 ± 0.2 min retention time and ≥ 95% AUC	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%
Purity	Reducing SDS-PAGE	≥ 95% monomer	Pass	96.1%	Pass	95.9%	Fail	94.2%	Fail	94%	Pass	96.4%	Fail	94.7%	Fail	93.8%
Physicochemical Properties	pH	7.4 ± 0.2	Pass	7.25	Pass	7.32	Pass	7.36	Pass	7.34	Pass	7.32	Pass	7.34	Pass	7.32

					Temperature 25 °C											
Test Parameter	**Test Method**	**Specification**	**0 months**		**1 month**		**3 months**		**6 months**		**9 months**		**12 months**		**24 months**	
Appearance	Visible appearance	Clear, colorless liquid, free of visible particles (CCFVP)	Pass	CCLFVP	Pass	CCLFVP	Pass	CCLFVP	Pass	CLFVP	Pass	CCLFVP	Pass	CCLFVP	Pass	CCLFVP
Identity	ESI-MS	12280 ± 3 Da	Pass	12280	Pass	12280	Pass	12281	Pass	12281	Pass	12281	Pass	12281	Pass	12281
Protein Concentration	A_{280}	1 ± 0.2 mg/mL	Pass	1.06	Pass	1.05	Pass	1.06	Pass	1.12	Fail	1.22	Fail	1.26	Fail	1.35
Potency	GM1-KDEL ELISA	EC50 shift < ±20% of reference standard	Pass	+2.85%	Pass	+2.56%	Pass	+8.53%	Pass	-1.7%	Pass	-5.3%	Fail	-38.8%	Fail	-26.1%
Purity	Size exclusion HPLC	16.4 ± 0.2 min retention time and ≥ 95% AUC	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	97.51%
Purity	Reducing SDS-PAGE	≥ 95% monomer	Pass	96.1%	Pass	96.2%	Fail	93.2%	Fail	93.1%	Fail	93.6%	Fail	91.5%	Fail	91.8%
Physicochemical Properties	pH	7.4 ± 0.2	Pass	7.25	Pass	7.41	Pass	7.32	Pass	7.34	Pass	7.31	Pass	7.37	Pass	7.25

3.2. Forced degradation stability assessment results

Forced Degradation Stability. The stability parameters used to evaluate EPICERTIN DS degradation products under forced degradation conditions included purity (SEC-HPLC and SDS-PAGE) and potency (GM1/KDEL ELISA) for all conditions previously described, with an additional identity (ESI-MS) assessment for the oxidation conditions. All specifications were the same as summarized in Table 1.

Purity. Forced degradation conditions were evaluated by SEC-HPLC and SDS-PAGE to assess purity. Conditions failing to meet specification by SEC-HPLC included high temperature (40 °C 48 hours, 40 °C 72 hours, 50 °C 24 hours, 50 °C 48 hours, 50 °C 72 hours, 60 °C 24 hours, 60 °C 48 hours, and 60 °C 72 hours) and low pH (pH 1 and pH 4), with representative chromatograms from each condition shown in Figure 5. All other conditions met specification. High temperature conditions generally showed both higher and lower molecular weight species on SEC-HPLC chromatograms, which could correspond to higher order structures (e.g., decamers and higher order aggregates) and lower order structures (e.g., monomer, dimer, etc.). Low pH conditions showed lower molecular weight species on SEC-HPLC chromatograms, which likely correspond to EPICERTIN monomer and dimer [66, 71].

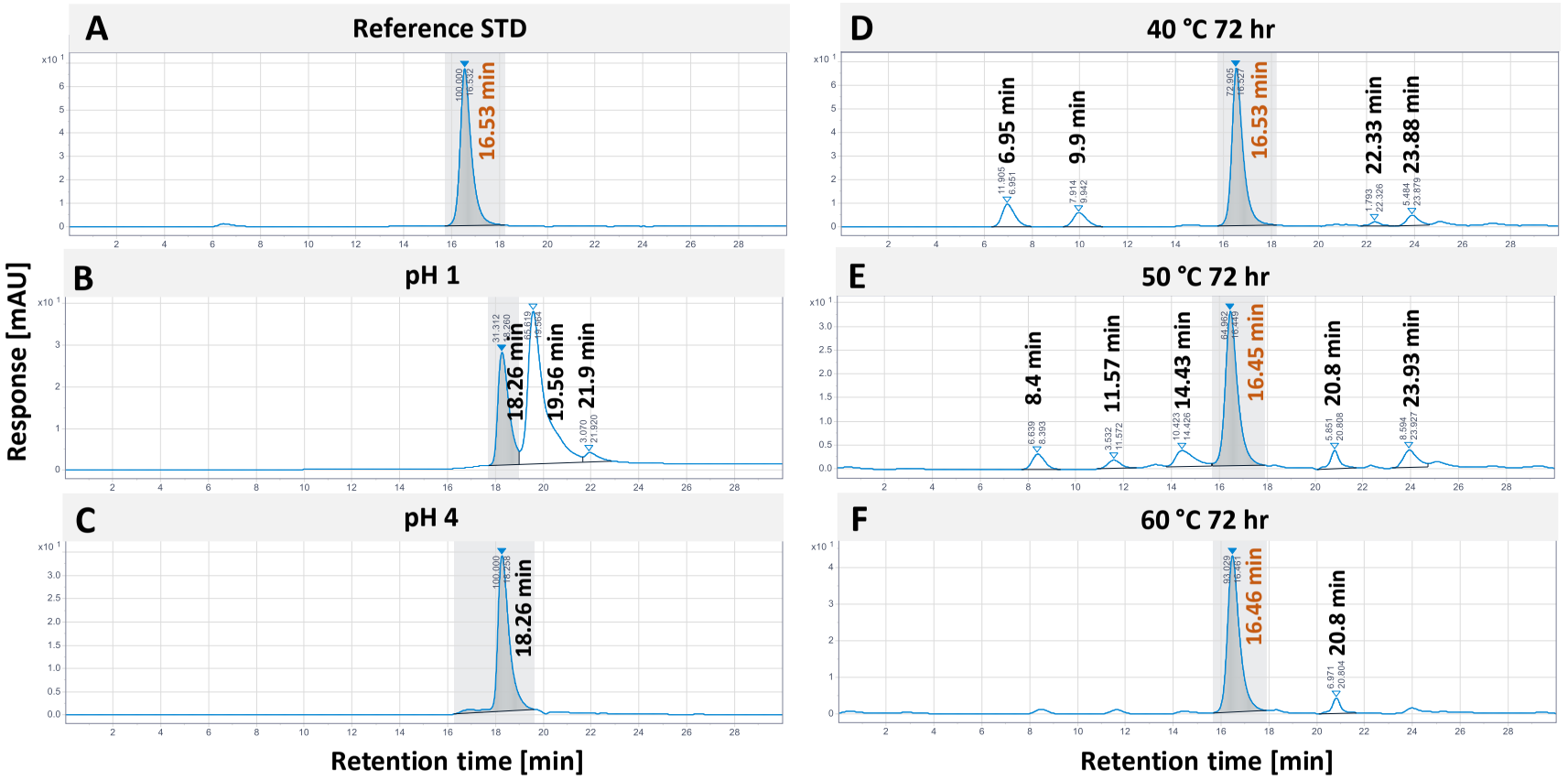

Figure 5. Forced degradation conditions which failed to meet specification by SEC-HPLC (purity). A) Reference standard. B-C) Low pH: pH 1 and pH 4, respectively. D-F) Representative chromatograms of high temperature conditions: 40°C 72 hours, 50°C 72 hours, 60°C 72 hours, respectively.

Upon performing this forced degradation assessment, we observed unique SEC-HPLC chromatogram peaks showing the presence of molecular weight species other than the expected native pentameric EPICERTIN species (Figure 5). To further characterize the purity of these species, we employed SDS-PAGE as an orthogonal method for all forced degradation conditions (Figure 6). Almost all conditions failed to meet the specification of $\geq$ 95% monomer on SDS-PAGE, with the exceptions of five: agitation (24 hours), high temperature (40 °C 48 hours), pH variation (pH 1), and oxidation (0.2% and 2% H_2O_2).

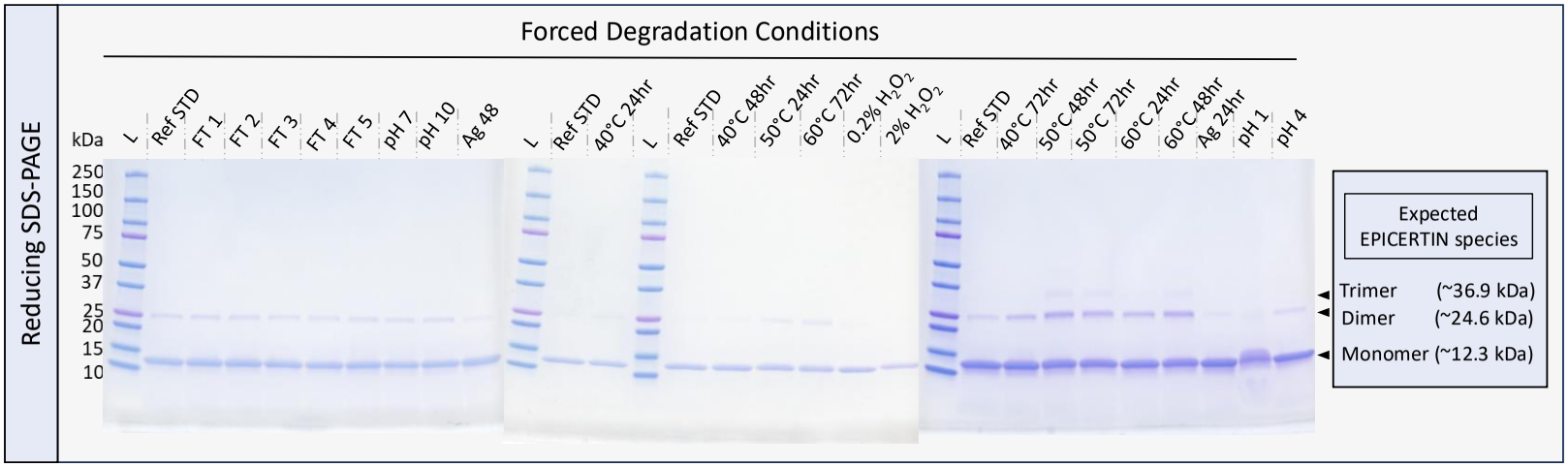

Figure 6. Forced degradation conditions on SDS-PAGE (purity). Reducing SDS-PAGE was employed as an orthogonal method of purity for all forced degradation conditions, in response to the presence of unique species of EPICERTIN observed on SEC-HPLC chromatograms. Abbreviations annotated on the gels are as follows: L (ladder, molecular weight standard), Ref STD (reference standard), FT (freeze thaw cycle), and Ag (agitation). The only conditions that met the specification of $\geq$ 95% monomer were agitation (24 hours), high temperature (40 °C 48 hours), pH variation (pH 1), and oxidation (0.2% and 2% H_2O_2). All other conditions failed to meet specification, caused by the presence of $\geq$ 5% EPICERTIN dimer and/or trimer content alongside the expected monomer. This finding suggests the incomplete denaturation of EPICERTIN into monomer under the forced degradation conditions.

Potency. Conditions failing to meet the GM1/KDEL ELISA specification included low pH (pH 1 and 4), which are shown in Figure 7. All other conditions met specification. GM1 binding was attenuated with pH 4 (+335.9% EC_{50} shift) and negligible with pH 1 (+34,128.5% EC_{50} shift).

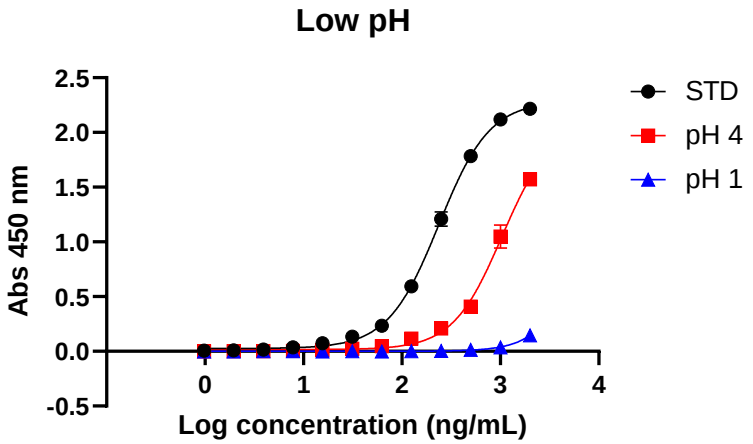

Figure 7. Forced degradation conditions which failed to meet specification by GM1/KDEL ELISA potency assessment. GM1 binding is attenuated with pH 4 (+335.9% EC_{50} shift) and negligible with pH 1 (4,128.5% EC_{50} shift).

Identity. ESI-MS was employed to monitor evidence of oxidation which occurs in mass-increase intervals of 16 Da [75, 82-84]. The lower strength oxidation condition, 0.2% H_2O_2 for 36 hours, met specification and remained unoxidized, however, the higher strength oxidation condition, 2% H_2O_2 for 36 hours, was sufficient to oxidize EPICERTIN DS as evidenced by a 16-17 Da increase in mass compared to an unoxidized reference standard (Figure 8A-B).

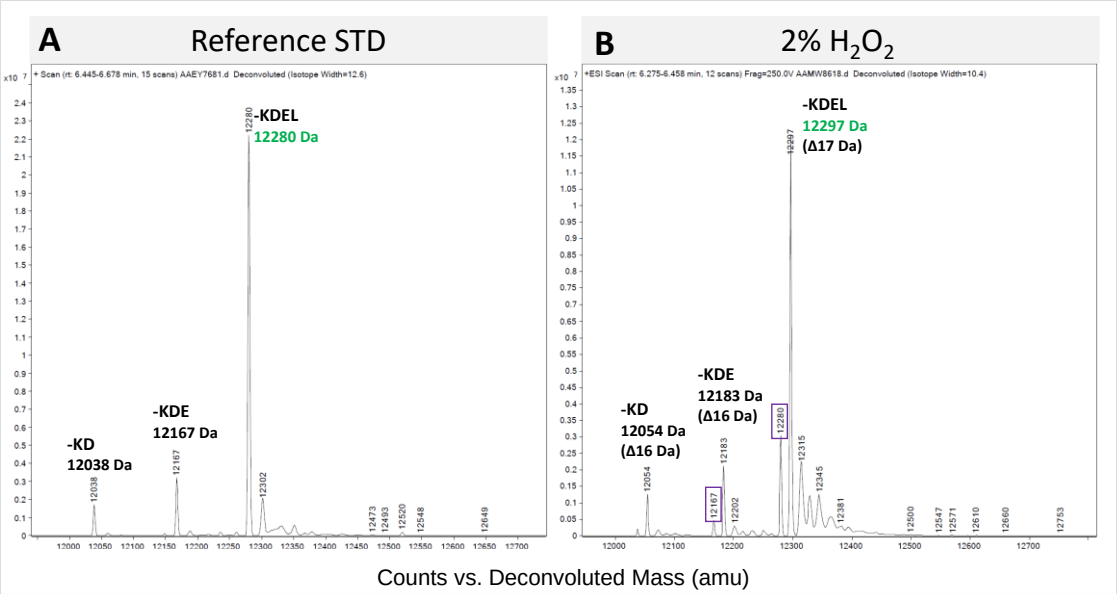

Figure 8. ESI-MS scan of oxidized EPICERTIN DS. A) Intact mass spec (MS) reference standard chromatogram demonstrating EPICERTIN identity within specification (12279.9 $\pm$ 3 Da) as well as expected -KD and -KDE truncated species. B) Oxidized EPICERTIN DS under 2% H_2O_2 treatment showing expected species albeit with 16 and 17 Da mass increases which indicate oxidation by addition of one oxygen atom. Residual unoxidized species are outlined in purple.

Overall, the results showed that EPICERTIN DS degraded under conditions of high temperature (above 40°C for 24 hours), as validated by SEC-HPLC (purity), as well as low pH (pH 1 and 4), as validated by SEC-HPLC and GM1/KDEL ELISA (potency). EPICERTIN DS was oxidized with 2% H_2O_2 as determined by ESI-MS. The complete forced degradation assessment is summarized in Table 4.

Table 4. Results summary of the forced degradation stability assessment

			High Temperature															
Test Parameter	**Test Method**	**Specification**	**40 °C 24 hr**		**40 °C 48 hr**		**40 °C 72 hr**		**50 °C 24 hr**		**50 °C 48 hr**		**50 °C 72 hr**		**60 °C 24 hr**		**60 °C 48 hr**		**60 °C 72 hr**	
Potency	GM1-KDEL ELISA	EC50 shift < ±20% of reference standard	Pass	-12.08%	Pass	2.14%	Pass	-6.49%	Pass	-6.74%	Pass	4.32%	Pass	-5.70%	Pass	1.54%	Pass	-8.48%	Pass	-4.51%
Purity	Size exclusion HPLC	16.4 ± 0.2 min retention time and ≥ 95% AUC	Pass	96.13%	Fail	68.32%	Fail	72.91%	Fail	58.23%	Fail	39.63%	Fail	64.96%	Fail	77.00%	Fail	74.25%	Fail	93.029%
Purity	Reducing SDS-PAGE	≥ 95% monomer	Fail	93.6%	Pass	97.7%	Fail	83.1%	Fail	89.2%	Fail	74.13%	Fail	77.6%	Fail	83.5%	Fail	76.3%	Fail	83.8%

			Agitation				Freeze Thaw Cycles									
Test Parameter	**Test Method**	**Specification**	**24 hr**		**48 hr**		**1**		**2**		**3**		**4**		**5**	
Potency	GM1-KDEL ELISA	EC50 shift < ±20% of reference standard	Pass	11.92%	Pass	7.21%	Pass	8.11%	Pass	5.22%	Pass	7.21%	Pass	13.27%	Pass	16.00%
Purity	Size exclusion HPLC	16.4 ± 0.2 min retention time and ≥ 95% AUC	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%
Purity	Reducing SDS-PAGE	≥ 95% monomer	Pass	98.2%	Fail	94.2%	Fail	81.1%	Fail	84.9%	Fail	85.3%	Fail	86.7%	Fail	85.3%

			pH								Oxidation			
Test Parameter	**Test Method**	**Specification**	**1**		**4**		**7**		**10**		**0.2% 36hr**		**2% 36hr**	
Potency	GM1-KDEL ELISA	EC50 shift < ±20% of reference standard	Fail	-162,343 %	Fail	-152.77 %	Pass	-3.93%	Pass	6.93%	Pass	5.00%	Pass	-10.48%
Purity	Size exclusion HPLC	16.4 ± 0.2 min retention time and ≥ 95% AUC	Fail	0.00%	Fail	0.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%	Pass	100.00%
Purity	Reducing SDS-PAGE	≥ 95% monomer	Pass	97.9%	Fail	94.16%	Fail	85.1%	Fail	82.4%	Pass	97.9%	Pass	97.7%
Identity	ESI-MS	12280 ± 3 Da	N/A								Pass	12280	Fail	12297

CHAPTER IV: DISCUSSION

The goal of this project is to advance the development of EPICERTIN as a novel mucosal healing biotherapeutic for IBD treatment. The stability assessments performed here were conducted on research-grade EPICERTIN DS and address the FDA requirement for the generation of CMC data, in order to facilitate a FIH clinical trial.

The long-term stability results indicate that the EPICERTIN DS, formulated as a 1 mg/mL solution in PBS, remains stable upon storage at 5°C up to 24 months. During storage at both 5 °C and 25 °C/ 60% RH, we observe alterations in monomeric content on SDS-PAGE at varied points throughout the study (Table 3). However, as observed by SEC-HPLC analysis, the native pentameric stability remains unchanged in all cases except for a slight (< 3%) degradation upon storage at ambient temperatures (25 °C/ 60% RH; Figure 3). Protein concentration was the only other parameter with noted deviation from specification for either temperature, with concentrations above specification beginning at the 12 month timepoint when stored at 5 °C and the 9 month timepoint when stored at 25 °C/ 60% RH. Further investigation revealed the concentration deviation stemmed from an inadequate container-closure system. Specifically, the lack of an airtight seal led to evaporation over time, causing a gradual increase in protein concentration. This insight informs future optimization of the formulation and its packaging to ensure consistent protein concentration throughout its shelf life, which

likewise ensures consistent potency. Overall, the EPICERTIN DS was demonstrated robust stability upon storage at 5 °C and showed the beginnings of degradation upon storage at 25°C/ 60% RH after 24 months.

In the forced degradation study, EPICERTIN DS degradation conditions were identified by SEC-HPLC [high temperature (40 °C 48 hours, 40 °C 72 hours, 50 °C 24 hours, 50 °C 48 hours, 50 °C 72 hours, 60 °C 24 hours, 60 °C 48 hours, and 60 °C 72 hours) and low pH (pH 1 and 4)], GM1/KDEL ELISA [low pH (pH 1 and 4)], and ESI-MS [oxidation (2% H_2O_2)]. Additionally, almost all forced degradation conditions failed to meet the specification of $\geq$ 95% monomer on SDS-PAGE.

In reducing SDS-PAGE, alongside EPICERTIN monomer, we sometimes observe a dimer and rarely trimer. This incomplete denaturation into monomer could be attributable to the unique biophysical behaviors of EPICERTIN DS degradation products. For instance, proteins are susceptible to secondary conformational changes or covalent protein modification induced by stress (e.g., thermal, high temperature, freeze/thaw, pH, etc.) that are caused by the same forces that drive protein folding (e.g. hydrogen, van der Waals bonding). These stress-induced conformational changes can promote aggregation [85-87], a major challenge for biopharmaceutical production [88]. Further, not all aggregates are created equally such that the formation of unique or heterogeneous aggregates, that possess distinct biochemical features and qualities, attributable to the particular type of stress, though the mechanisms are not fully understood [85]. The SDS-PAGE results of forced degradation conditions (Figure 6) show altered dissociation into EPICERTIN monomers, which could be one such altered biochemical quality caused by stress-induced secondary conformational changes. In the forced degradation study, SEC-

HPLC revealed high temperature formation of aggregates as well as the presence of lower molecular weight species, which could explain why these conditions failed SDS-PAGE. As for all other conditions that failed to meet specification for SDS-PAGE, it is plausible that the altered dissociation into monomers could also be explained by stress-induced changes that promote dissociation. We rely on SEC-HPLC and SDS-PAGE as orthogonal methods to evaluate purity as they complement each other by informing different aspects of purity. SEC-HPLC assessment informs the native conformation component of EPICERTIN DS purity. Although the pH 1 condition meets the SDS-PAGE specification of $\geq$ 95% monomer (97.9% monomer), it fails to meet the SEC-HPLC specification of $\geq$ 95% pentamer (0% pentamer), which is due to the fact that EPICERTIN dissociates into monomers under low pH conditions [47, 50]. Additionally, the forced degradation results suggest that SDS-PAGE is sensitive to altered dissociation into monomers, which could be evidence of stress-induced secondary changes. Interestingly, despite detecting new degradation patterns by the purity assessments, out of all forced degradation conditions tested, only low pH conditions (pH 1 and 4) affect potency as evidenced by the attenuated GM1 binding with the GM1/KDEL ELISA (Figure 7).

Of importance, understanding the implications of the presence of impurities and degradation products is crucial for the determination of how to manage them. For instance, the identification of degradation product comes with the establishment of acceptance criterion for a degradation product in a new drug substance or product. The ICH Q6A highlights the decision making in this process [89]. For example, for impurities in a drug substance, the process starts by identifying the impurity level in a sample. If the

measured impurity level is less than a predefined threshold, then it is deemed acceptable. However, if the level is higher than the threshold, an estimate is made to predict the maximum possible increase in impurity content by the retest date. This estimation is based on data gathered from stability studies which consider both accelerated and long-term storage conditions. If the estimated maximum impurity level remains acceptable, the batch is likely salvageable. Otherwise, the flowchart considers a qualified impurity level, which is a pre-determined acceptable amount of impurity. The maximum possible impurity level, including any anticipated degradation products, is then determined under appropriate storage conditions. This final level is then compared to the qualified level. If it exceeds the qualified level, the batch is rejected. On the other hand, if the maximum impurity level stays within the qualified level, the acceptance criterion is set to this new level, or a new qualified level is established altogether. Another example is for impurities in a drug product, where the following decision making flow applies to situations where degradation is identified during the manufacturing process. The first step involves estimating the maximum potential increase in the degradation product level based on existing batch data. If this estimated increase is determined to be acceptable, the maximum likely level, which considers the estimated increase and existing levels, is set as the acceptance criterion. However, if the initial estimation indicates an unacceptable increase, the flowchart diverges into two paths. One path involves comparing the maximum likely level of the degradation product to a qualified level, which is a pre-established acceptable amount. If the maximum likely level exceeds the qualified level, the batch is rejected. On the other hand, if the maximum likely level falls within the qualified threshold, two options are presented. The acceptance criterion can be set to this

new maximum likely level, or a completely new qualified level can be established to accommodate this specific degradation product.

Quantification of impurities and degradation products is employed to refine acceptance criteria. This approach ensures safety and efficacy are considered while setting these criteria. Evaluation of the EPICERTIN DS stability will be ongoing, with newly identified impurities and degradation products informing the generation of appropriate acceptance criteria. However, this necessitates the development of new, complementary analytical methods to address limitations of the primary panel of test methods. Notably, C-terminal truncation, a known impurity in plant-produced EPICERTIN DS, will be included in the drug substance and drug product specifications. Monitoring changes in the impurity profile will be a continuous process alongside verification of forced degradation results and expansion of the testing conditions.

The two-year stability study exemplifies the refinement of acceptance criteria. Initially, the SEC-HPLC data met the existing specification. However, column calibration prompted a redefinition of the acceptance criteria to ensure conformance with the specifications, as shown in Figure 3. Consequently, the reference standard is presented in Figure 3 instead of a comparison to the 0-month data point. This approach is acceptable because reference standards used to control impurities are evaluated and characterized according to their intended uses, and the drug substance itself can be used to estimate impurity levels [90].

In the future, we may explore effective protection of EPICERTIN DS from degradation for product development, if we find that further optimization is necessary

under specific circumstances. For instance, adequate protection from aggregation resulting from high temperature could be necessary. Mutagenesis and glycosylation modifications are protein engineering solutions employed in the context of mitigating immunogenicity caused by aggregation of therapeutic proteins [91]. Prevention of aggregation can also be accomplished through the use of excipients (e.g. salts, detergents, etc.). Also, we could explore the oxidation site and implement targeted interventions, such as a site-specific mutation, to protect against the potential for oxidation. For example, Q-GRFT, a clinically studied broad spectrum antiviral biotherapeutic, was protected from oxidation with a M78Q mutation in response to formulation stability assessments that revealed its potential to be oxidized at this site [92]. Oxidation of EPICERTIN DS is unexpected as EPICERTIN DS was not susceptible to oxidation during the two-year study nor were potency or purity affected by oxidation under forced degradation conditions (data not shown).

From a regulatory perspective, guidance authorities are interested in the identification of drug degradation products since such alterations in pharmaceutical quality attributes could cause changes in efficacy and safety profiles [75, 76]. EPICERTIN's "toolbox" provides orthogonal test methods which permit the assessment of DS stability over time and under forced degradation conditions. The forced degradation results identified conditions for which degradation products are produced, as determined by our stability indicating test methods, which thereby validates their stability-indicating power. These results also justify the use of these methods for the long-term stability study as well as for future clinical-grade EPICERTIN manufacturing processes. As in the case of EPICERTIN, all drugs on the path to the clinic should have a

tailored “toolbox” containing test methods capable of detecting degradation products to inform the drug’s stability and quality profiles, all of which are interrogated by regulatory agencies.

The current methods employed to assess the stability of EPICERTIN DS present limitations. Ongoing efforts aim to address these limitations and gaps in methodology. Discrepancies have been observed between methods, and some observations lack clear definitions in the context of the molecule. For example, ESI-MS uniquely identifies the content of C-terminally intact versus truncated species, a feature not assessed by other methods. While truncation goes undetected by SDS-PAGE, it does not appear to affect pentamer formation as observed by SEC-HPLC. Preliminary assessments using native PAGE (not shown here) suggest altered motility patterns based on C-terminal integrity. This method is under development for stability studies. The goal is to define and establish acceptance criteria for these parameters, enabling the monitoring of C-terminal truncation as an orthogonal approach to ESI-MS.

A limitation of SEC-HPLC is its sensitivity to higher molecular weight forms without definitive identification. Retention times for these species, as well as those of lower molecular weight forms like dimers, trimers, and tetramers, haven't been explicitly linked to their identities. The long-term and forced degradation studies have revealed previously unexplored discrepancies between assays, particularly between SEC-HPLC and reducing SDS-PAGE purity assessments. For example, SEC-HPLC might indicate a sample with 100% pentameric species, yet it might fail the reducing SDS-PAGE purity specifications (e.g., samples subjected to freeze-thaw 1 or pH 7 conditions). This

discordance suggests a need to revisit the suitability of the SDS-PAGE acceptance criterion and potentially modify it, as the implications of purity by SDS-PAGE require further investigation.

The forced degradation assessment, particularly the high temperature results, revealed an interesting observation. While all samples passed the potency assessment via the GM1/KDEL ELISA, they exhibited varying degrees of failure in the purity tests. This suggests the specific degradation products formed at high temperature do not impact the drug's potency. Alternatively, we have considered the possibility that the presence of 0.05% Tween 20 in the ELISA assay might artificially rescue the activity of degraded species. Future studies will investigate this phenomenon. Additionally, the development of an orthogonal potency bioassay is planned to strengthen the potency assessment. This new assay will utilize a more biologically relevant platform to directly evaluate the molecule's function.

In our ongoing stability assessments of research-grade EPICERTIN DS, we aim to build on this foundation to address limitations in the analytical methods and acceptance criteria as well as experimental design of the stability programs. We will expand on the long-term stability and forced degradation studies by implementing our full panel of methods for all conditions tested, as a limitation in the current designs lacked WB assessment at all timepoints in the long-term study as well as ESI-MS assessment for all forced degradation conditions. Expanding the analytical scope could help elucidate the reasons for observed discrepancies and potentially reveal new degradation patterns. For instance, the long-term stability study showed no change in C-

terminal truncation via ESI-MS (Figure 1). However, this aspect was not investigated in the forced degradation study, which could potentially explain non-passing results in the GM1/KDEL ELISA. The limitations identified during EPICERTIN's current development stage provide valuable insights that will be used to refine methodologies and establish more appropriate acceptance criteria. These advancements aim to achieve a deeper understanding of the molecule and inform more effective approaches in later development phases.

EPICERTIN is a promising first in class mucosal healing biotherapeutic with the potential to enhance the UC standard of care. As there are currently no FDA-approved drugs solely for this purpose, it has yet to be established whether such agents would be effective for all UC patients since their efficacy may depend on the underlying cause of inflammation in each individual case. However, mucosal healing is an important clinical goal for remission, and as an epithelial repair agent, EPICERTIN could serve as an earlier intervention option or complementary combination therapy to ultimately improve the long-term outcomes for patients. EPICERTIN could potentially be used earlier in the treatment course to promote healing and prevent disease progression. EPICERTIN might offer an alternative for patients who don't respond well to 5-ASAs or experience side effects from corticosteroids. EPICERTIN might also be used in combination with other medications like immunomodulators or anti-TNF drugs to achieve and maintain remission more effectively. Data on the long-term safety and efficacy of mucosal healing biotherapeutics has yet to be established, and more research is needed to understand the potential benefits and risks over extended use. EPICERTIN is currently undergoing preclinical development for UC, and following the establishment of a successful clinical

proof of concept, the potential for targeting CD can be further explored as well. The long-term stability and forced degradation assessments of EPICERTIN DS address key regulatory information required to prepare an investigational new drug (IND) submission to the FDA, which is the ultimate preclinical developmental milestone in EPICERTIN's path toward a FIH clinical trial for mucosal healing in IBD patients.

CHATPER V: CONCLUSIONS

The long-term stability and forced degradation assessments of research-grade EPICERTIN DS address key regulatory information required to prepare an investigational new drug (IND) submission to the FDA. EPICERTIN DS was subjected to an preliminary long-term stability program to evaluate its molecular stability and physicochemical properties. The stability of EPICERTIN DS, formulated in phosphate-buffered saline (PBS) at 1 mg/mL and stored at 5°C and 25°C/60% RH, was monitored over a 2-year period according to relevant regulatory guidelines. Evaluations of EPICERTIN DS at 0, 3, 6, 9, 12, and 24 months included assessment of purity by SDS-PAGE and size exclusion high performance liquid chromatography (SEC-HPLC), identity by electrospray ionization mass spectrometry (ESI-MS) intact mass analysis, and potency by GM1-binding KDEL-detection ELISA (GM1/KDEL ELISA). Additionally, a forced degradation study was conducted to identify the EPICERTIN DS degradation products under various conditions including thermal stress, pH variations, agitation, and oxidation. The degradation patterns were analyzed by assessing purity (using SEC-HPLC and SDS-PAGE), potency (via GM1/KDEL ELISA) for all conditions as well as intact mass (via ESI-MS) only for oxidized samples. The results indicate that EPICERTIN DS maintains robust stability upon storage at 5 °C and showed the beginnings of degradation upon storage at 25°C/ 60% RH after 24 months. The forced degradation study effectively identified degradation products, particularly under conditions of high temperatures (above 40°C for 24 hours), low pH values (pH 1 and 4), and oxidation upon exposure to

2% H_2O_2, among others. These findings highlight EPICERTIN's robust long-term stability in PBS formulation, as well as the stability indicating power of the analytical assays implemented. However, discordancy in the results of our stability studies necessitate improvements in our future assessments.

Future development of EPICERTIN for mucosal healing in IBD will necessitate ongoing evaluation of its stability to understand the interpretation and identification of new impurities and degradation products, which will then inform the evolution and establishment of appropriate acceptance criteria. To address limitations in the current primary analytical methods, the development of new, complementary methods will be crucial. For example, we are interested in implementing a reverse phase (RP)-HPLC method to identify charge variants, as well as native PAGE as an orthogonal method to assess C-terminal intactness, and lastly a bioassay as an orthogonal method to assess potency. The information gathered from these continued efforts will guide future characterization needs as EPICERTIN approaches later phases of development.

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CURRICULUM VITA

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EDUCATION & TRAINING:	B.S. Molecular Biotechnology Western Kentucky University 2016-2021 B.S. Biochemistry Western Kentucky University 2016-2021

PROFESSIONAL SOCIETIES:	Association for Women in Stem (AWIS) 2018 – Present American Chemical Society (ACS) 2023 – Present

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AWARDS:	First Place Session Winner (2023) UofL Brown Cancer Center Research Retreat, poster presentation over “Therapeutic effects of EPICERTIN in an oxazolone colitis model”. Integrated Program in Biomedical Sciences (IPIBS) Fellowship, UofL School of Medicine (2021-2023) Cum Laude, WKU Honor’s College graduate (2021) WKU Honors Thesis: Pass with Distinction (2021)

Thesis: *Development of a DNA Biosensor Using CRISPR/dCas9 on a Graphene Oxide Surface for Detecting Antibiotic Resistance Genes*.

First Place Session Winner (2019)
Southeast Regional IDEA Conference, poster presentation in Bioengineering, Biotechnology, and Nanotechnology session over "Novel application of 2D nanosheet and DNA binding proteins for detecting antibiotic resistance genes".

Gatton Academy Graduate Research and Experiential Learning Award (2019)

First Place Session Winner (2018)
Kentucky Academy of Sciences Regional Research Conference, poster presentation in Botany over "Cultivation of Peanuts in Oak Tree Saw Dust to Increase Resveratrol".

Gatton Academy Graduate Research and Experiential Learning Award (2018)

Selected for a National Science Foundation's International Research Experiences for Students (NSF IRES) training opportunity (2018)

Citation of Research Achievement (2018)
Recognition for research presentation at Posters-at-the-Capitol, awarded by Representative Chad McCoy and the Kentucky House of Representatives.

Gatton Academy Research Supplies Grant (2018)

Horatio Alger Association State Scholar (2018)
The Horatio Alger Association honors the achievements of outstanding individuals in our society who have succeeded in spite of adversity and who are committed to supporting young people in pursuit of increased opportunities through higher education.

First Place Session Winner (2017)

Kentucky Academy of Sciences Research Conference, oral presentation over “A Study of Mercury in Bald Eagle Feathers and Quills”.

First Place Session Winner (2017)
WKU Research Conference, oral presentation over “A Study of Mercury in Bald Eagle Feathers”.

WKU Sisterhood Research Internship Grant (2017)
Awarded funding through the WKU Sisterhood for summer research with the WKU Chemistry Department

Gatton Academy Research Supplies Grant (2017)

Gatton Academy Research Supplies Grant (2016)

LOCAL/REGIONAL MEETINGS:

Poster Presentation, “Therapeutic effects of EPICERTIN in an oxazolone colitis mouse model,” UofL Brown cancer Center Research Retreat, September 2023.

Poster Presentation, “Therapeutic effects of EPICERTIN in an oxazolone colitis mouse model,” UofL Inflammation and Pathogenesis Colloquium, April 2023.

Oral Presentation, “Stability Assessment of Epicertin, a Biopharmaceutical Candidate for Mucosal Healing,” UofL Graduate Student Regional Research Conference, March 2023.

Poster Presentation, “Biochemical Characterization of Epicertin Variants for Investigating Wound Healing Mechanisms,” Research! Louisville, September 2022.

Poster Presentation, “Novel dsDNA Biosensor Utilizing Zinc Finger Proteins on 2D Graphene Oxide Surface for Detecting Antibiotic Resistance Genes,” WKU Virtual Student Research Conference, May 2020.

Poster Presentation, “Zinc finger proteins on graphene oxide surface for detecting antibiotic resistance genes,” KY NSF EPSCoR Annual Super Collider Conference, Lexington, KY, February 2020.

Poster Presentation, “Novel application of 2D nanosheet and DNA binding proteins for detecting antibiotic resistance genes,” Southeast Regional IDEA Conference, Louisville, KY, November 2019.

Poster Presentation, “Development of a Double-Stranded DNA Biosensor Using Engineered Zinc Finger Protein Pairs Linked to a Full β-lactamase for Detecting Antibiotic Resistance Genes,” WKU Student Research Conference, March 2019.

Poster Presentation, “Cultivation of Peanuts in Oak Tree Saw Dust to Increase Resveratrol,” Kentucky Academy of Science Annual Meeting, November 2018.

Oral Presentation, “A Study of Mercury in Bald Eagle Feathers and Quills,” WKU Student Research Conference, March 2018.

Poster Presentation, “A Study of Mercury in Bald Eagle Feathers and Quills,” Posters-at-the-Capitol, February 2018.

Oral Presentation, “A Study of Carbon Storage in a Costa Rican Cloud Forest,” Research Showcase in Costa Rica, January 2018.

Oral Presentation, “A Study of Mercury in Bald Eagle Feathers and Quills,” Kentucky Academy of Sciences, November 2017.

Oral Presentation, “A Study of Mercury in Bald Eagle Feathers,” WKU Student Research Conference, March 2017.

NATIONAL MEETINGS:

Poster Presentation, “Developing a double-stranded DNA biosensor using engineered zinc finger proteins linked to a β-lactamase for detecting antibiotic resistance genes,” American Chemical Society, Orlando, FL, April 2019.