

# Interventions with Potential to Mitigate Injection Site Reactions Following Subcutaneous Elamipretide Administration: A Phase 1, Crossover Study

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## Abstract

**Background and Purpose:** Elamipretide is a mitochondrial-targeting agent being developed for the treatment of mitochondrial dysfunction-associated diseases. While prior studies have shown that subcutaneous elamipretide is generally safe/well tolerated, injection site reactions (ISRs) were reported in most subjects. We evaluated the efficacy of interventions to mitigate ISRs, identify underlying ISR mechanisms, and evaluate the pharmacokinetic and safety profile of subcutaneous elamipretide. **Experimental Approach:** Subcutaneous elamipretide 60 mg was administered to healthy subjects (N=10) on six separate occasions with/without potential ISR interventions (mometasone furoate, ice application, tacrolimus ointment, doxepin cream, and oral diphenhydramine). ISR clinical/self-assessments, blood samples, and safety data were collected at predetermined intervals. Pre-clinical studies investigated mast cell-specific receptor MRGPRX2 mediation of ISRs. **Key Results:** Mometasone significantly reduced the incidence of induration/swelling and pruritus. Diphenhydramine significantly decreased the incidence of induration; 50% reported somnolence. Ice application significantly reduced the incidence of pain, although it reduced elamipretide's maximum plasma concentration and area-under-the-curve from time 0-6hrs versus elamipretide alone. Preclinical data suggest that SQ-elamipretide induced ISRs by activating MRGPRX2 in humans and its ortholog Mrgprb2 in mice. **Conclusion and Implications:** Elamipretide activated MRGPRX2 and Mrgprb2 receptors, resulting in activation of mast cells and inflammation in mouse models, suggesting that targeting mast-cell activation may ameliorate elamipretide ISRs. Topical mometasone prior to subcutaneous elamipretide demonstrated significant reductions in ISR signs and symptoms and did not cause significant changes in elamipretide plasma exposure or additional adverse events. Therefore, mometasone prior to subcutaneous injection of elamipretide warrants further investigation in clinical studies for alleviating ISRs.

## Interventions with Potential to Mitigate Injection Site Reactions Following Subcutaneous Elamipretide Administration: A Phase 1, Crossover Study

**Shortened Title:** Interventions to Mitigate Injection Site Reactions from Elamipretide

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**Conflicts of interest:** SCB, AS, LEK, and AA are employees of Stealth BioTherapeutics (Newton, MA)

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**Ethics approval:** The clinical trial was conducted in accordance with international ethics guidelines, including the Declaration of Helsinki, Council for International Organizations of Medical Sciences International Ethical Guidelines, ICH GCP guidelines, and all applicable laws and regulations. The study was approved by institutional review boards, and all participants provided written informed consent. Animal studies were conducted in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals.

**Abstract** << 250/250 words>>

**Background and Purpose:** Elamipretide is a mitochondrial-targeting agent being developed for the treatment of mitochondrial dysfunction-associated diseases. While prior studies have shown that subcutaneous elamipretide is generally safe/well tolerated, injection site reactions (ISRs) were reported in most subjects. We evaluated the efficacy of interventions to mitigate ISRs, identify underlying ISR mechanisms, and evaluate the pharmacokinetic and safety profile of subcutaneous elamipretide.

**Experimental Approach:** Subcutaneous elamipretide 60 mg was administered to healthy subjects (N=10) on six separate occasions with/without potential ISR interventions (mometasone furoate, ice application, tacrolimus ointment, doxepin cream, and oral diphenhydramine). ISR clinical/self-assessments, blood samples, and safety data were collected at predetermined intervals. Preclinical studies investigated mast cell-specific receptor MRGPRX2 mediation of ISRs.

**Key Results:** Mometasone significantly reduced the incidence of induration/swelling and pruritus. Diphenhydramine significantly decreased the incidence of induration; 50% reported somnolence. Ice application significantly reduced the incidence of pain, although it reduced elamipretide's maximum plasma concentration and area-under-the-curve from time 0-6hrs versus elamipretide alone. Preclinical data suggest that SQ-elamipretide induced ISRs by activating MRGPRX2 in humans and its ortholog Mrgprb2 in mice.

**Conclusion and Implications:** Elamipretide activated MRGPRX2 and Mrgprb2 receptors, resulting in activation of mast cells and inflammation in mouse models, suggesting that targeting mast-cell activation may ameliorate elamipretide ISRs. Topical mometasone prior to subcutaneous elamipretide demonstrated significant reductions in ISR signs and symptoms and did not cause significant changes in elamipretide plasma exposure or additional adverse events. Therefore, mometasone prior to subcutaneous injection of elamipretide warrants further investigation in clinical studies for alleviating ISRs.

**Keywords:** Elamipretide, Injection Site Reaction, mitigation, mometasone, safety, tolerability, MRGPRX2

## Introduction

Elamipretide is an aromatic-cationic tetrapeptide that readily penetrates the cell membrane and transiently localizes to the inner mitochondrial membrane (IMM) where energy (adenosine triphosphate [ATP]) production occurs, thereby improving mitochondrial function by restoration of the physical and biochemical properties of the IMM through its reversible association with cardiolipin (CL), a phospholipid that is unique to mitochondrial membranes (Szeto, 2008; Birk et al., 2013; Grazioli & Pugin, 2018; Mitchell et al., 2020). Specifically, this association improves membrane stability, enhances ATP synthesis in several organs including the heart, kidney, neurons, and skeletal muscle, and reduces reactive oxygen species production (Zhao et al., 2005; Manczak et al., 2010; Szeto & Schiller, 2011; Dai et al., 2013; Siegel et al., 2013; Birk et al., 2014; Brown et al., 2014; Eirin et al., 2014; Nickel et al., 2014; Szeto & Birk, 2014; Alam et al., 2015; Roshanravan et al., 2021). Elamipretide has been extensively examined in multiple preclinical studies and clinical trials for diseases involving mitochondrial dysfunction, consistently demonstrating amelioration of pathologic symptoms, including improvement in skeletal muscle strength, cardiac stroke volume, and kidney function (Manczak et al., 2010; Birk et al., 2013; Dai et al., 2013; Siegel et al., 2013; Eirin et al., 2014; Stauffer et al., 2016; Daubert et al., 2017; Saad et al., 2017; Karaa et al., 2018; Sabbah et al., 2019; Allen et al., 2020; Reid Thompson et al., 2021).

The investigational agent, elamipretide, is being developed for the treatment of patients with a variety of diseases, such as Barth syndrome (BTHS) and Primary Mitochondrial Myopathy (PMM), among others, in which genetic abnormalities affecting the mitochondria lead to life-long symptoms that require long-term administration of elamipretide (Karaa et al., 2018; Sabbah et al., 2019; Karaa et al., 2020; Reid Thompson et al., 2021). Clinical development of elamipretide has focused on subcutaneous (SC) administration. Prior studies have shown that SC elamipretide up to 80 mg once daily is generally safe and well tolerated, although most longer-term studies have used SC elamipretide 40 or 60 mg once daily [data on file, Stealth BioTherapeutics Inc.]. However, injection site reactions (ISRs) were reported in the majority of subjects receiving treatment. In multiple-dose clinical trials lasting >8 days, ISRs were relatively common in subjects: injection site erythema (47%), pruritus (45%), pain (22%), induration (19%), swelling (14%), urticaria (13%), bruising (12%), hemorrhage (6%), and mass (6%). Although these ISRs are typically mild in nature and resolved within 4 hours of elamipretide administration, they can lead to subject withdrawal or discontinuation during chronic daily administration [data on file, Stealth BioTherapeutics Inc.].

The Mas-related G-protein coupled receptor member X2 (MRGPRX2), which is expressed almost exclusively by mast cells that populate connective tissues, including the skin (Tatemoto et al., 2006; Motakis et al., 2014), has received increasing attention in recent years for its link to ISRs (McNeil 2021a). Mast cell activation in the skin causes immediate inflammation, and many therapeutic drugs associated with high frequencies of ISRs have been shown to activate MRGPRX2 directly, and thus trigger inflammation via mast cells. Notably, MRGPRX2 is preferentially activated by drugs with cationic and aromatic properties (Grimes et al., 2019), and since elamipretide has these properties, it is highly plausible that elamipretide is an MRGPRX2 agonist. While the current management of ISRs consists of alternation of daily injection sites around abdominal quadrants, interventions that target mast cell activation or the effects of mast-cell-derived mediators warrant investigation. To improve patient comfort, the aim of the present study was to evaluate the efficacy of potential interventions used to mitigate elamipretide-induced ISRs, identify any role of the MRGPRX2 receptor in elamipretide ISRs, and further understand the PK and safety of 60 mg SC elamipretide administration. The agents chosen for study (mometasone, tacrolimus, doxepin, diphenhydramine) have dosing regimens supported by the label for the product and are supported by their ability to reduce inflammatory responses by targeting mast cell activation.

## Methods

### *Study Design*

This was a Phase 1, open-label, 4-week, 6-part, crossover study. After a screening period (up to 28 days), a total of ten eligible subjects received SC elamipretide 60 mg (0.75 mL) to alternating quadrants of the

abdomen on each of six occasions with or without potential ISR interventions. The first treatment arm (Arm 1) started on Day 1 with the SC administration of elamipretide only followed by the remaining treatment arms (Arms 2-6) with the following interventions sequentially. On Day 3, Arm 2 received mometasone furoate 0.1% ointment applied once to an area ~8 cm in diameter and covered with a hydrocolloid occlusive dressing (DuoDERM<sup>®</sup> Extra Thin) prior to SC elamipretide administration 1 week later on Day 10. Arm 3 received ice application to an area ~10 cm in diameter around the injection site 5 minutes before and 5 minutes after elamipretide administration on Day 12. Arm 4 received tacrolimus 0.1% ointment applied to an area ~10 cm in diameter around the injection site 15 minutes before elamipretide administration on Day 14. Arm 5 received doxepin 5% cream applied to an area ~10 cm in diameter around the injection site 15 minutes before elamipretide administration on Day 16. Arm 6 received diphenhydramine 50 mg orally taken 2 hours prior to elamipretide administration on Day 18. All treatments were administered by and/or under the supervision of investigative staff, ensuring compliance. The drug classes and/or mechanisms of action of these ISR interventions are summarized in Table 1. All patients participated in all arms of the study and therefore, randomization and blinding were not applicable.

For each treatment arm, blood samples for the determination of plasma concentrations of elamipretide and its metabolites were collected pre-dose and at 0.25, 0.5, 1, 2, 4, and 6 hours after elamipretide administration. Vital signs were recorded pre-dose and at 0.5 and 6 hours after elamipretide administration. ISR clinical and self-assessments were performed before and at 0.5, 1, 2, 4, 6, 12, 24, and 48 hours after elamipretide administration. Injection sites were photographed at 0.5, 1, 2, 4, and 12 hours after elamipretide administration.

This study was conducted in accordance with international ethics guidelines, including the Declaration of Helsinki, Council for International Organizations of Medical Sciences International Ethical Guidelines, International Conference on Harmonisation Good Clinical Practice guidelines, and all applicable laws and regulations. The protocol was approved by the institutional review boards and all participants provided written informed consent prior to entering the trial.

### *Participants*

Healthy subjects [?]18 and [?]65 years of age were enrolled with body mass index [?]18.5 and [?]32.0 kg/m<sup>2</sup> and body weight [?]120 kg. Participants had to be willing and able to provide consent and adhere to trial requirements. All participants of childbearing potential were on acceptable birth control. The main exclusion criteria were estimated creatinine clearance <90 mL/min, history of clinical hypersensitivity or allergy to any pharmaceutical agent, chronically administered antihistamines or corticosteroids within the last 28 days, and significant mental illness. Tattoos, scarring, or other abdominal skin characteristics which could confound assessment of ISRs were also considered exclusion criteria.

### *Study Assessments*

The primary endpoints of this study comprised the following efficacy measures: clinical assessments and subject self-assessments. Specifically, the primary endpoints consisted of comparisons of the grading of each ISR parameter following administration of elamipretide with each separate intervention versus the grading of each ISR parameter following administration of elamipretide alone. For clinical assessments, the clinical staff used a standard procedure adapted from the Division of Aids Table for Grading the Severity of Adult and Pediatric Adverse Events to score pain, erythema, induration/swelling, and pruritus using a 4-point scale based on severity (1 = mild, 2 = moderate, 3 = severe, and 4 = potentially life threatening). The self-assessments were based on a questionnaire to determine how bothered the patient was following each injection of elamipretide and included the following parameters: pain, burning sensation, cold sensation, itching, redness, swelling, and bruising (Not at all, A little, Moderately, Very, Extremely).

The secondary endpoints of this study consisted of PK and general safety assessments. Plasma samples were analyzed for elamipretide and its M1 and M2 metabolites using a validated liquid chromatography/tandem mass spectrometry assay. The lower limits of assay quantitation were 3.0, 1.5, and 1.0 ng/mL for elamipretide,

M1, and M2, respectively. Maximum plasma concentration ( $C_{\max}$ ) and area under the plasma concentration-time curve from time zero to 6 hours ( $AUC_{0-6h}$ ) were calculated using Phoenix WinNonlin<sup>(r)</sup> software.

Safety assessments consisted of comparison of treatment-emergent adverse events (TEAEs) reported following administration of elamipretide with each separate intervention versus TEAEs reported following administration of elamipretide alone. Safety measurements for determination of TEAEs included routine clinical laboratory tests, 12-lead ECG, physical examination, and vital signs. TEAEs were graded with respect to severity (mild, moderate, or severe) and relationship to study drug (unrelated or unlikely, possible, or probably related). Injection sites were photographed at 0.5, 1, 2, 4, and 12 hours post-elamipretide-dose for qualitative purposes.

### *Data and Statistical Analysis*

All statistical analyses were performed using SAS GRID Linux/SAS Studio (Version 9.4, or higher). PK parameters for elamipretide and its metabolites, M1 and M2, were summarized using descriptive statistics. Comparisons between treatments (elamipretide with each separate intervention versus elamipretide alone) were evaluated by an analysis of the log-transformed PK parameters ( $C_{\max}$  and  $AUC_{0-6h}$ ) by performing an analysis of variance with treatment effects. From these analyses, least square means (LSMs), least square treatment differences, and 90% confidence intervals (CIs) for the treatment differences on log-scale were obtained. The results were transformed back to the original scale by exponentiation to provide treatment geometric LSM, point estimates of the geometric test (elamipretide alone)/reference (elamipretide with each separate intervention) LSM ratios, 90% CI, for these ratios, and p-values. Chi-square test or Fisher exact test were used to examine the differences in ISR responses between treatment arms at each timepoint. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. The thresholds for statistical significance and trends toward significance were defined as  $p < 0.05$  and  $p < 0.20$ , respectively.

### *Preclinical MRGPRX2, Mrgprb2, Mast Cell Activation, and Evans Blue Assays*

Calcium mobilization in heterologous cells - Clonal HEK293 cells expressing human MRGPRX2 and Galpha15, created in the lab, were plated in wells of a 96-well plate, loaded with Fluo-4 acetoxymethyl ester (Fluo-4 AM) for 45 minutes at 37degC and allowed to rest for 30 minutes before use, as previously described (McNeil et al., 2015). 2X elamipretide was added by manual pipetting at designated time points, and fluorescence intensity before, during, and after elamipretide incubation was measured with a confocal microscope using the FITC filter. A similar protocol was used for MRGPRX2-transfected Chem-1 cells and their parental cell line, Chem-1. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose medium (4.5 g/L) with 10% fetal bovine serum, non-essential amino acids, and HEPES. Geneticin (G418) was used to maintain receptor expression in the MRGPRX2 cell line. These cells were seeded to 96-well plates, loaded with Fluo-4 AM for 30 minutes at 37degC, washed, and allowed to rest for 30 minutes before use. Cells were resuspended in 50  $\mu$ L of PBS with calcium and magnesium and 50  $\mu$ L of 2X elamipretide or ionomycin were added to stimulate the cells. A no-drug vehicle control and ionomycin positive control were also included. Plates were read every six seconds for two minutes using a Biotek plate reader.

EC<sub>50</sub> calculation - Clonal HEK293 cells expressing human MRGPRX2 and Galpha15 were plated in a 96-well plate, loaded with Fluo-4 AM for 45 minutes at 37°C, and allowed to rest for 30 minutes before use. The assay was performed using a fluorescent plate reader and baseline fluorescence was calculated as the average of a 30 second read. 2X elamipretide was added manually after baseline recordings, and response was defined as the maximum signal within 90 seconds after addition of elamipretide minus the baseline fluorescence signal. Concentrations were tested in duplicate, the assay was performed six times, and the curve was calculated as a four-parameter non-linear fit with variable slope.

Peritoneal mast cell activation assay - Primary peritoneal mast cells were isolated from wild type and Mrgprb2 knockout mice (McNeil et al., 2015). These mice were selected because Mrgprb2 is the mouse ortholog of MRGPRX2. Mast cells were incubated in DMEM and supplemented with 10% fetal bovine serum and 100 ng/mL human stem cell factor for 2 hours in a 96 well plate. The cells were then loaded with Fluo-4 AM for

30 minutes at room temperature, washed, and allowed to rest for 30 minutes before use. Using a fluorescence microscope, free intracellular calcium levels in each mast cell were measured. If the Fluo-4 signal rose by at least 50% for [?]10 seconds, cells were identified as responding.

Evans Blue assay - Anesthetized wild type and Mrgprb2 knockout mice up to 8 months of age were injected intravenously with 50  $\mu$ L of 12.5 mg/mL Evans Blue in saline, followed by injection of 5  $\mu$ L of 0.5 mg/mL elamipretide in one paw and saline in the other paw. Mice were sacrificed 15 minutes after elamipretide injection and paw tissue was collected, dried for 24 hours at 50°C, and weighed. Evans Blue was extracted by a 24-hour incubation in formamide at 50°C and the optical density was read at 600 nm and 740 nm using a spectrophotometer. The value at 740 nm was subtracted from the value at 600 nm to attain the final readout.

### *Materials*

Elamipretide 60 mg was provided by Stealth BioTherapeutics (Needham, MA, USA) as 0.75 mL SC injections. Mometasone ointment 0.1% (NDC 68462-225-55) was manufactured by Glenmark Pharmaceuticals Limited and DuoDEM Extra Thin dressing (product number 187955) was manufactured by Convatec. The suppliers for tacrolimus ointment 0.1% (NDC 0168-0416), doxepin cream 5% (NDC 0378-8130-45), and diphenhydramine 25 mg tablets (NDC 50580-226-51) were Fourgera Pharmaceuticals Inc., Mylan Pharmaceuticals Inc., and Johnson & Johnson Consumer Inc, respectively. Fluo-4 AM (LOT#2566212) and eBioScience cell stimulation cocktail 500x (REF 2024-04-18) containing ionomycin were supplied by Invitrogen by Thermo Fisher Scientific. DMEM high glucose medium (4.5 g/l) (LOT#2472338), 10% fetal bovine serum, non-essential amino acid solution (REF 1140-050, LOT#2301965), HEPES (1M) stock (REF 15630-080, LOT#2192897), and active Geneticin (G418) 50 mg/mL (REF 101315, LOT#2389171) were supplied by Gibco. Evans blue in saline and 100 ng/mL human stem cell factor were supplied by Sigma. MRGPRX2-transfected Chem-1 cells and their parental cell line, Chem-1 cells, were obtained through Eurofins Discovery.

## **Results**

### *Participants*

Of the 13 subjects screened, 10 individuals underwent treatment and all completed the study. Of the 10 participants, 50% were male. Mean (SD) age was 40.7 (5.89) years, weight 75.31 (11.35) kg, height 167.98 (9.25) cm, and body mass index 26.58 (2.92) kg/m<sup>2</sup>. All participants were White with 90% classified as Fitzpatrick skin type III (darker white [tans after initial burn]) and 10% as Fitzpatrick skin type IV (light brown [burns minimally, tans easily]).

### *Efficacy outcomes*

With respect to clinical assessment of ISR signs and symptoms, mometasone significantly ( $p=0.0031$ ) reduced the incidence of induration/swelling at 0.5 hours post-elamipretide dose (Table 2). There were trends to significance in reduction of induration/swelling at 1 hour post-dose ( $p=0.0736$ ) and pruritus at 0.5 hours post-dose ( $p=0.0573$ ) with mometasone (Tables 2 and 3). Diphenhydramine significantly ( $p=0.0198$ ) decreased the incidence of induration/swelling at 1 hour post-elamipretide dose with a trend to significance ( $p=0.0698$ ) in reduction of induration/swelling at 0.5 hours post-dose (Table 2). With respect to subject-reported assessment of ISR signs and symptoms, ice application significantly ( $p=0.0325$ ) reduced the incidence of pain at 0.5 hours post-elamipretide dose (Table 4) and there was a trend to significance ( $p=0.0573$ ) for reduction of itching at 0.5 hours post-elamipretide dosing (Table 5). With mometasone, there were trends to significance to reductions in the incidence of swelling at 1 hour post-elamipretide dose ( $p=0.3698$ ), bothersome itching at 1 hour post-dose ( $p=0.1409$ ), and increased redness at 12 hours post-dose ( $p=0.0867$ ): the latter believed to be attributable to a reaction to the occlusive dressing. Tacrolimus and doxepin demonstrated no significant differences in ISR signs and symptoms compared to elamipretide administered alone in all clinical and self-assessments.

The injection site photographs collected aligned with the signs and symptoms of ISRs commonly described following administration of elamipretide by SC injection. In addition, the photographs supported the clinical

assessments of the ISRs that were conducted at the same timepoints. Overall, the ISRs captured on the photographs were resolving at 4 hours post-elamipretide dose with the exception of bruising, which appeared to form after the 12-hour post-dose timepoint in those affected. In the mometasone arm, the photographs aided in deciphering erythema grading in subjects where the pattern of redness appeared to be related to the use of the occlusive dressing and not the injection.

### *Pharmacokinetics*

In all study arms where elamipretide was administered with a topical or systemic drug, elamipretide AUC<sub>0-6h</sub> was not significantly different compared to that after administration of elamipretide alone. Ice application arm demonstrated a statistically significant impact on elamipretide C<sub>max</sub> and AUC<sub>0-6h</sub> compared to elamipretide alone. Specifically, mean (SD) C<sub>max</sub> of elamipretide was reduced by ~23% (1693.0±369.23 vs 2213.0±634.54 ng/mL, p=0.0003) and the AUC<sub>0-6h</sub> of elamipretide was reduced by ~12% (4747.8±818.99 vs 5401.3±1096.06 ng/mL\*h, p<0.0001). Ice application arm showed a statistically significant reduction of C<sub>max</sub> (407.9+-81.72 vs 454.1+-70.24 ng/mL, p=0.0028) and AUC<sub>0-6h</sub> (1849.1+-352.12 vs 2079.1+-278.81 ng/mL\*h, p<0.0001) for the M1 metabolite and AUC<sub>0-6h</sub> (210.7+-32.65 vs 239.4+-27.09 ng/mL\*h, p=0.0135) for the M2 metabolite.

### *Safety*

Apart from data relating to ISRs, which were not captured as AEs because they were the designated study endpoints, few TEAEs were reported in this study on daily SC elamipretide doses of 60 mg. The only TEAE observed in more than one subject was that of somnolence in the diphenhydramine treatment arm, where five subjects (50%) reported mild somnolence. Although it is reasonable to suspect that night-time administration of diphenhydramine could reduce the occurrence of somnolence, doing so was not a part of the study protocol. Therefore, a conclusions cannot be drawn here. There were no apparent differences between any treatment arms with respect to vital signs, ECG parameters, or laboratory values.

### *Preclinical MRGPRX2, Mrgprb2, and Mast Cell Data*

We examined whether ISRs may be caused by activation of mast cells through MRGPRX2. First, we found that HEK293 cells stably transfected with MRGPRX2 showed massive calcium mobilization – a key component of MRGPRX2-mediated intracellular signaling – after incubation with elamipretide, as assessed by an increase in fluorescence intensity of the calcium sensitive dye Fluo-4 (Figure 1A). Untransfected cells did not demonstrate calcium mobilization. This was also observed in Chem-1 cells transfected with MRGPRX2 (Figure 2A), but not untransfected cells (Figure 2B), confirming that the response was due to MRGPRX2 and that elamipretide is an MRGPRX2 agonist. Quercetin, proposed to antagonize MRGPRX2 signaling (Ding et al., 2019), inhibited elamipretide activation of MRGPRX2 (Figure 2C). Neither M1 (Figure 2D) or M2 metabolites (Figure 2E) elicited calcium mobilization in MRGPRX2 cells, suggesting that only the parent compound is responsible for mast cell activation. Using the HEK293 cell line, we established that the half maximal effective concentration (EC<sub>50</sub>) was 63 +- 13 µg/ml (Figure 1B), well under the 80 mg/ml injection concentration used for human administration and suggesting that elamipretide readily activates skin mast cells at this concentration. Next, we found that elamipretide also activated Mrgprb2, the mouse ortholog of MRGPRX2 (Figure 3), and triggered calcium mobilization in wild type but not Mrgprb2 knockout primary mouse peritoneal mast cells, demonstrating that mast cell activation was Mrgprb2-specific (Figure 3B). Finally, we used the Evans Blue assay to examine skin inflammation after *in vivo* administration of elamipretide. Evans Blue is a dye that binds to albumin in the blood; intravenous injection of Evans Blue thus labels albumin blue, and when albumin-containing fluid escapes from the bloodstream and into tissues after mast cell degranulation, the tissue becomes filled with dye. We found that SC administration of elamipretide into wild type mouse hindpaws triggered immediate tissue swelling and fluid extravasation, reminiscent of ISRs, and that this was reduced by ~80% in Mrgprb2 knockout mice, demonstrating that most of the reaction was due to Mrgprb2 activation of mast cells (Figure 4).

## **Discussion**

The aim of this preliminary phase 1 study was to evaluate the potential efficacy of interventions that might be

used to mitigate ISRs following the SC administration of 60 mg elamipretide in healthy subjects as well as any potential impact on PK and safety. We focused on treatments that target mast cells or factors upstream or downstream to their activation, due to our preclinical data that strongly suggest that elamipretide-associated ISRs are caused by direct activation of mast cells through MRGPRX2. Overall, mometasone appeared to favorably mitigate the presumed pseudo-allergic reactions observed following SC elamipretide, specifically reducing pruritus and induration/swelling with no effect on PK. Ice application ameliorated early injection site pain and itching, but reduced the absorption of elamipretide, while diphenhydramine demonstrated potential in reducing induration/swelling but caused sedation in some cases. Tacrolimus and doxepin, as utilized in this study, demonstrated little impact on reported ISRs compared to elamipretide alone and are not likely to be recommended for ISR mitigation, though additional investigation could be considered. There were no significant changes in elamipretide or M1 and M2 metabolite plasma exposures with any of the treatments except for ice application, which reduced exposure to elamipretide and its metabolites.

Although ice application and diphenhydramine showed some reduction in ISR signs and symptoms, mometasone revealed the most promise. The reduction in pruritus and induration/swelling demonstrated by mometasone enabled patients to be more comfortable with the elamipretide treatment. In addition, mometasone may further improve elamipretide tolerability by reducing scratching and subsequent scratching-related skin damage. In some subjects, the hydrocolloid occlusive dressing applied over the mometasone ointment resulted in redness of the covered area (i.e. the injection site area), confounding the ability to identify erythema due to ISRs. The capture of ISR photographs proved useful in deciphering erythema grading in subjects where the pattern of redness appeared to be related to the use of the occlusive dressing. However, it is possible that erythema as an ISR was overreported in the mometasone treatment arm of this study.

Despite ice application and diphenhydramine reducing some ISR signs and symptoms, both interventions presented undesirable effects. Specifically, the simple intervention of ice application reduced early injection site pain and itching, but reduced the  $C_{max}$  and  $AUC_{0-6h}$  of elamipretide and its metabolites (M1 and M2), possibly due to vasoconstriction. Neither M1 nor M2 are biologically active and the potential reduction in plasma concentrations of these metabolites is not therefore anticipated to have a clinical impact [data on file, Stealth BioTherapeutics Inc.]. However, while the potential ramification on efficacy of the disruption in absorption of the active parent drug is unclear, lowering plasma exposure of elamipretide is not desirable, making ice application a less appropriate mitigation option. Similarly, diphenhydramine showed some potential in reducing induration/swelling, but a significant incidence (50%) of mild somnolence was reported in this treatment arm. While second generation antihistamines were not included in this study, the observations with diphenhydramine suggest that other antihistamines that are less sedating could provide utility in mitigating ISRs.

Based on the outcomes of mometasone and antihistamine use, further investigation is warranted to include separate and combined interventions with oral second-generation antihistamines (such as fexofenadine) and topical mometasone without the use of an occlusive dressing. Fexofenadine is a selective peripheral H1 receptor antagonist that does not readily cross the blood-brain barrier, causing less drowsiness in comparison to the first-generation antihistamines, such as diphenhydramine. Several international dermatology and allergy organizations issued a common guideline on chronic urticaria management, recommending the regular use of second-generation antihistamines as first-line treatment. Fexofenadine appears safe and well tolerated and daily doses can be titrated upwards in case of no improvement (Zuberbier, 2018). Chronic urticaria could be considered as the closest model to chronic ISRs in terms of available data on therapeutic management. With regard to mometasone, given that elamipretide is injected daily at alternating sites, applications with mometasone twice a day (one prior and one post-injection) appear to be a more practical alternative to the use of mometasone with occlusive dressing.

Our focus on mast cells arose from preclinical experiments which established that elamipretide, in addition to its intended function, also acts as an agonist of the human G protein-coupled receptor MRGPRX2. MRGPRX2 is primarily expressed by mast cells, which are constitutive residents of the skin and other tissues, trigger rapid tissue inflammation, and mediate many of the symptoms of allergic diseases. We reasoned that

MRGPRX2 might be involved because it can be activated by many cationic peptides and small molecules with properties similar to elamipretide and which also cause ISRs and other pseudo-allergic reactions (McNeil 2021a). We have several pieces of evidence to support the hypothesis that elamipretide-associated ISRs are due to MRGPRX2 activation. First, elamipretide triggers intracellular signaling pathways, as measured by calcium mobilization, in cell lines forced to express MRGPRX2 or its mouse ortholog *Mrgprb2*, but not in unmodified cell lines that do not natively express the receptors (Figures 2 A, B). Notably, this could be blocked by a molecule reported to inhibit MRGPRX2 signaling (Figure 2C). MRGPRX2 activation by elamipretide is physiologically relevant, as the calculated  $EC_{50}$  is over 1,000-fold lower than the injection concentration in our clinical trial. Second, elamipretide causes intracellular signaling, again as measured by calcium flux, in primary mouse mast cells from wild type but not *Mrgprb2* knockout mice (Figure 3). Third, SC elamipretide injection into mouse hindpaws triggered rapid swelling via fluid extravasation from the bloodstream, similar to human ISRs, in wild type mice, while extravasation was nearly absent in mice lacking *Mrgprb2* (Figure 4). These effects almost certainly are due to the parent drug, as elamipretide's metabolites have no effect on MRGPRX2 signaling (Figures 2 D, E).

While itch produced in humans by injection of MRGPRX2 agonists can be blocked by antihistamines (Hasbak et al., 2006), development of inhibitors is still in its infancy with no candidates in clinical trial yet (McNeil, 2021a). Indeed, a combination of H1 and H2 histamine receptor antagonists have demonstrated efficacy in blocking MRGPRX2-driven systemic and local reactions (McNeil 2021b) and could be considered in future elamipretide-driven ISR studies. Although the topical steroid mometasone reduced pruritus and induration/swelling following SC elamipretide in this study, its anti-inflammatory mechanism is unclear but is thought to act by inhibition of the arachidonic acid pathway (Spada, et al., 2018; ELOCON<sup>®</sup> Prescribing Information, 2018). In addition to anti-inflammatory effects, topical steroids, such as mometasone, possess anti-mitotic, immunosuppressive, and vasoconstrictive effects (Gabros et al., 2021), which may have played a role in mitigating ISR signs in this study.

Overall, the data collected in this study support prior findings that SC elamipretide is generally safe and well-tolerated (Karaa et al., 2020; Thompson et al., 2021; Mettu et al., 2022). With the exception of data relating to ISRs, very few TEAEs were identified in this study. The only TEAE seen in more than one subject was that of somnolence in the diphenhydramine treatment arm. Given that somnolence is a well-known side effect of diphenhydramine (Sicari & Zabbo 2021), this adverse event was considered likely related to diphenhydramine and not to elamipretide treatment.

A limitation of the study was the relatively low number of subjects evaluated (N=10), which impacted the potential to demonstrate statistically significant results with respect to efficacy of mitigation. This study was not powered to show a statistically significant difference in the ISR profile between treatment arms but was meant to identify signals that could warrant further investigation in other clinical settings.

## Conclusions

Application of topical mometasone prior to SC elamipretide administration demonstrated a significant reduction in ISR signs induced by elamipretide and did not cause significant changes in elamipretide plasma exposure or additional adverse events. Further investigation of mometasone is warranted for mitigation of elamipretide-induced ISRs and improving compliance. Treatment options that target mast cell activation may ameliorate elamipretide ISRs and should also be explored in future studies.

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## Figures

### Figure 1. Responses of MRGPRX2-Expressing HEK293 Cells to Elamipretide

A. Representative Fluo-4 fluorescence traces from HEK293 cells stably expressed with MRGPRX2 or its mouse ortholog Mrgprb2. Arrows indicate when elamipretide was added. Each trace is from a different cell in the same imaging field. A rise in fluorescence indicates receptor activation. B. Responses of MRGPRX2-expressing HEK293 cells to elamipretide (labeled as “SBT”) at the indicated concentrations.

**Figure 2:** Elamipretide, but not M1 and M2 metabolites, elicits intracellular calcium mobilization in a dose- and MRGPRX2-dependent manner

**Panel A:** Elamipretide induced calcium mobilization in MRGPRX2 cells. **Panel B:** Elamipretide does not elicit calcium mobilization in the parental cell line, Chem-1. **Panel C:** Quercetin inhibited elamipretide activation of MRGPRX2. **Panels D and E:** Neither M1 (**D**) or M2 metabolites (**E**) elicited calcium mobilization in MRGPRX2 cells.

**Figure 3.** Mast cells are activated by elamipretide in an Mrgprb2-dependent manner

A. Representative images of Fluo-4 fluorescence changes in response to elamipretide (labeled “SBT”). Fluorescence signals were converted to a heat map for ease of visualization. B. Average responses of wild type or Mrgprb2 knockout primary mouse peritoneal mast cells to 500 micrograms/ml elamipretide. (n=3 mice for each genotype, > 200 mast cells counted for each genotype, p < 0.01, t-Test, Two-Sample Assuming Unequal Variances).

**Figure 4:** In vivo inflammation in mice is induced by elamipretide in an Mrgprb2-dependent manner

Left panel: Evans Blue extravasation in wild type (WT) and Mrgprb2 knockout (KO) mice. Left paws injected with 0.5 mg/mL elamipretide/right paws injected with a saline control. Right, quantification of Evans Blue accumulation, measured as specific optical density (OD) per gram of tissue, in the footpads. Responses were calculated as the OD in elamipretide-injected paws, divided by the OD in saline-injected paws. (n=7 for wild type and n=8 for knockout mice. P < 0.01, t-Test, Two-Sample Assuming Unequal Variances) Elamipretide induced strong Evans Blue labeling and saline induced much less (left), dependent on Mrgprb2, as elamipretide induced almost no Evans Blue accumulation in Mrgprb2 knockout mice (left). Measurements of Evans Blue accumulation in footpads revealed that accumulation was ~ 80% less in Mrgprb2 knockout mice, demonstrating that at this concentration, most of the inflammation was dependent on Mrgprb2.

**Bullet point summary:**

What is already known:

The mitochondrial targeting agent, elamipretide, consistently improves pathologic symptoms in subjects with mitochondrial disease.

Elamipretide is safe and well-tolerated; injection site reactions are the most commonly reported adverse event.

What this study adds:

Elamipretide activated MRGPRX2 receptors, suggesting that therapeutically targeting mast cell activation may ameliorate elamipretide ISRs.

Mometasone significantly reduced the incidence of induration/swelling and pruritus following subcutaneous administration of elamipretide.

Clinical Significance:

Mometasone showed promise in mitigating elamipretide-induced injection site reactions and further investigation is warranted.

Treatment options that target mast cell activation may ameliorate elamipretide ISRs and should be explored.

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