Molecular Therapy
Nucleic Acids
Original Article

Novel readthrough agent suppresses nonsense mutations and restores functional type VII collagen and laminin 332 in epidermolysis bullosa

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Recessive dystrophic epidermolysis bullosa (RDEB) and junctional epidermolysis bullosa (JEB) are lethal blistering skin disorders resulting from mutations in genes coding for type VII collagen (*COL7A1*) and laminin 332 (*LAMA3*, *LAMB3*, or *LAMC2*), respectively. In RDEB, 25% of patients harbor nonsense mutations causing premature termination codons (PTCs). In JEB, a majority of mutations in *LAMB3* are nonsense mutations (80%). ELX-02, an aminoglycoside analog, has demonstrated superior PTC readthrough activity and lower toxicity compared to gentamicin in various genetic disorders. This study investigated the ability of ELX-02 to suppress PTCs and promote the expression of C7 and laminin 332 in primary RDEB keratinocytes/fibroblasts and primary JEB keratinocytes harboring nonsense mutations. ELX-02 induced a dose-dependent production of C7 or laminin β3 that surpassed the results achieved with gentamicin. ELX-02 reversed RDEB and JEB cellular hypermotility and improved poor cell-substratum adhesion in JEB cells. Importantly, ELX-02-induced C7 and laminin 332 localized to the dermal-epidermal junction. This is the first study demonstrating that ELX-02 can induce PTC readthrough and restore functional C7 and laminin 332 in RDEB and JEB caused by nonsense mutations. Therefore, ELX-02 may offer a novel and safe therapy for RDEB, JEB, and other inherited skin diseases caused by nonsense mutations.

INTRODUCTION

Epidermolysis bullosa (EB) comprises a group of skin fragility disorders that lead to the development of blisters, erosions, and scarring of varying severity upon minor physical contact.[1] Recessive dystrophic EB (RDEB) is inherited in an autosomal recessive manner and is caused by a mutation in the *COL7A1* gene. This genetic alteration leads to a deficiency or total absence of type VII collagen (C7).[2] In normal human skin, fibroblasts and keratinocytes produce C7. C7 forms anti-parallel dimers that aggregate together to create anchoring fibrils (AFs), which play a crucial role in anchoring the epidermis to the dermis at the dermal-epidermal junction (DEJ).[3] The diminished dermal-epidermal adherence observed in RDEB patients frequently leads to fibrosis-related complications.[4] RDEB patients typically have a limited life expectancy, with most patients eventually succumbing to aggressive squamous cell carcinomas.[5] While numerous treatments, such as gene therapy, protein therapy, and cell therapy, are under development for RDEB, the existing treatment approaches primarily focus on providing supportive care to limit wound formation and secondary infection.[6] A localized therapy using topical herpes simplex virus type 1 (HSV-1)-based gene therapy to restore C7 and improve wound healing in RDEB patients was recently approved by the US Food and Drug Administration.[7]

Junctional EB (JEB) is caused by mutations in the *LAMA3*, *LAMB3*, and *LAMC2* genes, resulting in decreased or absent production of laminin 332, a heterotrimeric molecule crucial to the formation of anchoring filaments.[8,9] Laminin 332 plays a critical role in maintaining dermal-epidermal adherence.[10] JEB patients suffer from extensive blistering of the skin and mucocutaneous regions, resulting in chronic infections, difficulty feeding, and refractory anemia.[11] Similar to RDEB, therapeutic options for severe JEB are limited. However, various potential treatments have been suggested, including protein replacement therapy,[12] gene therapy,[13] and bone marrow transplantation.[14,15] In contrast to patients with RDEB, patients with severe JEB rarely survive beyond the first year of life.[16]

The EB phenotype can be attributed to various mutations. Approximately 25% of RDEB patients are estimated to carry a nonsense mutation. *LAMB3* mutations account for 80% of patients with severe JEB, and ∼95% of severe JEB-associated *LAMB3* mutations are nonsense mutations.[11,17] Nonsense mutations generate premature termination codons (PTCs), resulting in an unstable mRNA transcript that is either degraded or translated into a truncated and non-functional polypeptide.[18] Recent studies have demonstrated that aminoglycoside antibiotics, such as gentamicin, are able to suppress PTCs in several genetic disorders caused by nonsense mutations.[10] We have previously

Received 12 April 2024; accepted 5 September 2024;
https://doi.org/10.1016/j.omtn.2024.102334.

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demonstrated that gentamicin has the ability to induce PTC readthrough and production of full-length C7 or laminin 332 in cultured RDEB and JEB cells.[10,19] We also previously showed that topical and short-term intravenous gentamicin restored C7 or laminin 332 in EB patients' skin and improved wound healing.[20–22]

Aminoglycosides have been linked to nephrotoxicity and ototoxicity, particularly with higher doses and prolonged use.[23,24] Consequently, investigators are developing compounds that can mimic the function of aminoglycosides in inducing PTC readthrough while simultaneously minimizing toxicity. ELX-02 is a non-antibiotic aminoglycoside analog specifically designed to enhance PTC readthrough, leading to increased protein production while simultaneously mitigating adverse effects.[25] This compound operates by selectively targeting eukaryotic ribosomes, thereby reducing its impact on eukaryotic mitochondria, which is considered a major factor in the toxicity associated with traditional aminoglycosides.[25,26] Furthermore, studies have demonstrated that ELX-02 exhibits fewer adverse effects compared to its aminoglycoside derivative counterparts.[27–29] ELX-02 has shown efficacy in stimulating protein production from PTC mutations without disrupting native stop codons or normal physiological protein synthesis.[30] To date, studies have shown that ELX-02 exhibits effectiveness in both *in vivo* and *in vitro* studies, specifically as a potential treatment for other diseases arising from nonsense mutations such as cystic fibrosis and cystinosis.[27,31,32] ELX-02 was found to be well tolerated with adequate bioavailability in phase 1 clinical trials.[25,28] Interestingly, while gentamicin is associated with nephrotoxicity, ELX-02 is undergoing phase 2 clinical trials to address renal pathologies like nephropathic cystinosis and Alport syndrome, underscoring the reduced toxicity profile of ELX-02.[27,32,33]

In this study, we sought to determine the feasibility of using ELX-02 in inducing PTC readthrough and restoring C7 and laminin 332 in RDEB and JEB cells harboring nonsense mutations. We showed that ELX-02 induced a dose-dependent increase in the production of full-length C7 in primary RDEB fibroblasts and keratinocytes. Additionally, ELX-02 induced the production of laminin 332 in primary keratinocytes cultured from two JEB patients. ELX-02-induced C7 or laminin 332 also corrected the abnormal cellular phenotypes of RDEB and JEB cells and incorporated into the DEJ.

RESULTS

The ability of ELX-02 to produce full-length C7 in RDEB fibroblasts is dose dependent

To assess the feasibility of ELX-02 as a potential treatment for RDEB resulting from nonsense mutations, we conducted experiments using different concentrations of ELX-02 and compared its effectiveness to gentamicin in primary RDEB fibroblasts derived from two RDEB patients with nonsense mutations, RDEB1 and RDEB2. RDEB1 is homozygous for R578X mutations, whereas RDEB2 is heterozygous for R613X and R1683X mutations. RDEB fibroblasts were incubated in increasing concentrations of ELX-02, and cell lysates were then prepared and subjected to immunoblot analysis. As shown in Figures 1A and 1B, ELX-02 induced the production of the full-length, 290-kDa C7 α chain in a dose-dependent manner. When compared to gentamicin, the highest concentrations (200 μM) of ELX-02-treated cells exhibited significantly more C7 protein production than gentamicin at the previously determined optimal dose.[19] Untreated parent cells showed no or minimal C7 expression. The optimal concentration of ELX-02 that resulted in the highest readthrough and full-length C7 production was 200 μM in both patient cells. At the optimal concentration of 200 μM, quantification showed that ELX-02 restored full-length C7 to more than 4.1- and 3.3-fold higher levels compared to those induced by gentamicin for RDEB1 and RDEB2, respectively. Importantly, the level of C7 expression achieved was 126.6% and 56.9% of that seen in normal human fibroblasts (NHFs) for RDEB1 and RDEB2, respectively. Cellular cytotoxicity was not observed under any of the ELX-02 concentrations tested above (Figure S1). Thus, these results indicate that ELX-02 induces dose-dependent PTC readthrough of nonsense mutations in RDEB fibroblasts.

The ability of ELX-02 to produce full-length C7 in RDEB keratinocytes is dose dependent

Both dermal fibroblasts and epidermal keratinocytes are responsible for the production of C7. To determine whether ELX-02 can also induce PTC readthrough and C7 production in RDEB keratinocytes, we used primary keratinocytes from the same two RDEB patients with nonsense mutations (RDEB1 and RDEB2). As shown in Figures 1C and 1D, similar to the results from RDEB fibroblasts, treatment of these cells with ELX-02 resulted in a dose-dependent production of full-length C7, with the highest C7 production occurring at 100 μM. At the optimal dose of 100 μM, ELX-02 induced full-length C7 to levels 4.0–4.8 times higher than those observed in RDEB keratinocytes treated with optimized gentamicin. The level of C7 expression achieved was 33.3% and 24.1% of that seen in normal human keratinocytes (NKCs) for RDEB1 and RDEB2, respectively. Cellular cytotoxicity was not observed under any of the ELX-02 concentrations tested above (Figure S2). These results indicate that ELX-02 can induce PTC readthrough and restore C7 production in both primary RDEB fibroblasts and keratinocytes.

Production of full-length C7 in RDEB fibroblasts and keratinocytes increases with multiple doses of ELX-02

To better reflect clinical therapeutic use, we sought to determine the effects of continued dosing with ELX-02 on the production of C7 in primary RDEB fibroblasts and keratinocytes. RDEB1 (fibroblasts) and RDEB2 (keratinocytes) cells were incubated with growth medium that received daily supplementation with 50 μM ELX-02 for 5 consecutive days. As shown in Figures 2A and 2B, RDEB1 fibroblasts and keratinocytes displayed a progressive increase in C7 production with successive doses of ELX-02. No apparent toxicity was observed (Figure S3). These data demonstrate that multiple doses of ELX-02 induce increasing amounts of PTC readthrough, and thus C7 production, in RDEB fibroblasts and keratinocytes.

ELX-02 mediates dose-dependent induction of laminin 332 in JEB keratinocytes

Since the majority of the mutations that cause JEB are nonsense mutations, we sought to explore whether ELX-02 was as effective in JEB

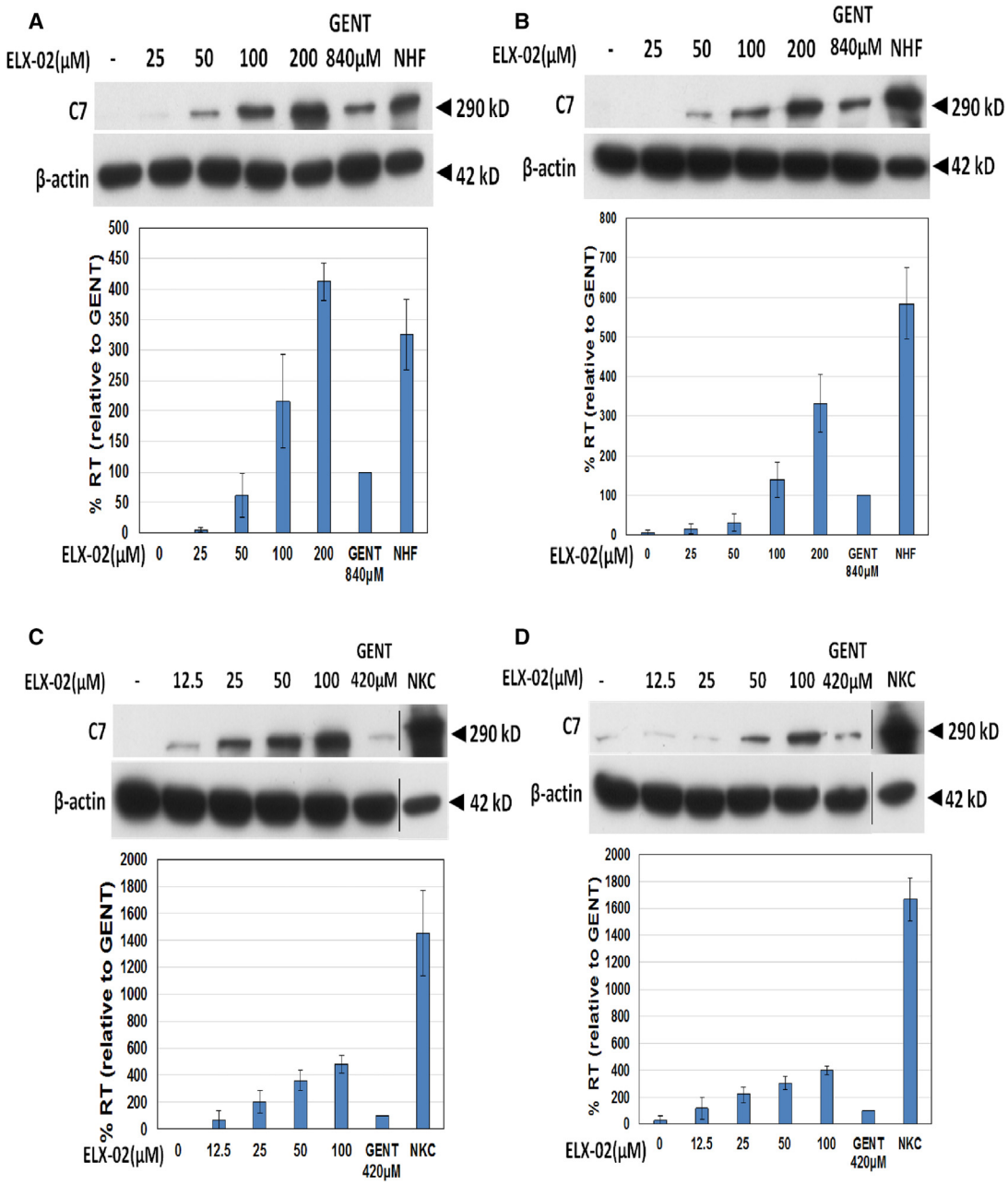

Figure 1. ELX-02 mediated dose-dependent induction of full-length C7 production in RDEB fibroblasts and keratinocytes

Primary RDEB fibroblasts, denoted as RDEB1 (A) and RDEB2 (B), and primary RDEB keratinocytes, identified as RDEB1 (C) and RDEB2 (D), were treated with increasing concentrations of ELX-02 and gentamicin (GENT) as indicated, for 48 h. Cell lysates were prepared and then subjected to 4%–12% SDS-PAGE, followed by immunoblot analysis with a rabbit polyclonal antibody to the NC1 domain of C7 or anti-β-actin (loading control) antibody. Note that ELX-02 induced full-length C7 production in a dose-dependent manner in RDEB fibroblasts and keratinocytes. ImageJ analysis of C7 expression normalized with β-actin is shown below the respective blots. The results are displayed as a percentage of the level of C7 obtained from cells treated with gentamicin alone (100%). We performed three independent experiments, and similar results were obtained (see Figure S6). Error bars, SE of three different experiments. Solid black line indicates where the gel is cropped from the same blot. RT, readthrough.

keratinocytes as it was in RDEB cells. We used primary keratinocytes from two JEB patients (JEB1 and JEB2) with nonsense mutations in *LAMB3* to test the effects of ELX-02 on PTC readthrough and laminin β3 production. JEB1 is heterozygous for C325X/c.629-12T>A, and JEB2 is heterozygous for R42X and R635X mutations. Cells were incubated with increasing doses of ELX-02 for 48 h, and then cellular extracts were prepared and subjected to immunoblot analysis. As shown in Figures 3A and 3B, treatment of both JEB cells with

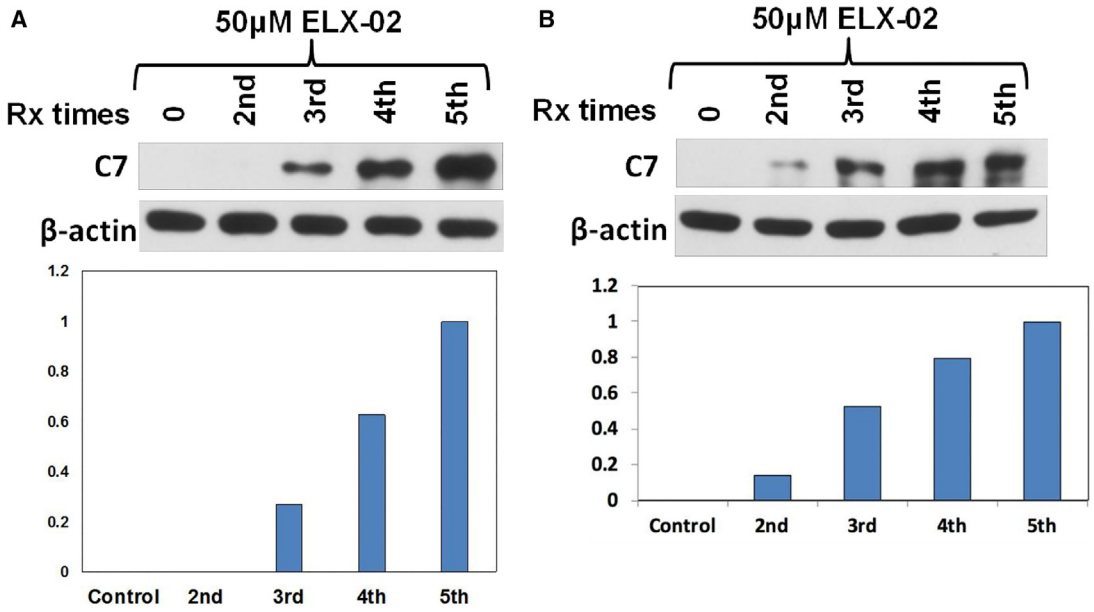

Figure 2. ELX-02-induced production of full-length C7 increased with continued dosing

RDEB1 fibroblasts (A) and RDEB1 keratinocytes (B) were incubated with growth media in the absence of ELX-02 or were given consecutive daily treatments (Rx) of ELX-02 for up to 5 days. Cell lysates were prepared and then subjected to 4%–12% SDS-PAGE, followed by immunoblot analysis with a rabbit polyclonal antibody to the NC1 domain of C7 or anti-β-actin (loading control) antibody. The results are displayed as a fraction of the level of C7 obtained from the fifth dose of ELX-02. Note that ELX-02 induced full-length C7 expression that increased with daily treatment.

ELX-02 resulted in a dose-dependent production of full-length, 140-kDa laminin β3. In contrast, untreated cells displayed no or minimal laminin β3 expression. The optimal, nontoxic ELX-02 concentration that maximized readthrough and full-length laminin β3 production was 100 μM in both cell types. Under this optimized concentration, quantification showed that ELX-02 induced full-length laminin β3 expression to levels 4.9–10.4 times higher than those observed in JEB keratinocytes treated with optimized gentamicin. Importantly, the level of laminin β3 expression achieved was 183.3% and 56.6% of that seen in NKCs for JEB1 and JEB2, respectively. We did not observe any cellular cytotoxicity under any of the ELX-02 concentrations tested above (Figure S4). These data indicate that ELX-02 induces PTC readthrough capable of producing full-length laminin-β3.

ELX-02 corrects the hypermotility characteristic of RDEB fibroblasts

The suppression of PTCs is mediated by a mispairing between the stop codon and near-cognate aminoacyl tRNA.[34,35] Although PTC readthrough can induce the production of a full-length protein, the resultant protein may have a different amino acid substituted in place of the original amino acid, which can alter the structure or function of the resultant protein product.[34,35] We have previously shown that RDEB fibroblasts demonstrate hypermotility compared with NHFs.[36] To test the functionality of ELX-02-induced C7, we conducted a fibroblast migration assay to assess the motility of both treated and untreated RDEB fibroblasts. Figure 4A demonstrates representative microscopic fields of NHFs and RDEB1 and RDEB2 primary fibroblasts untreated or treated with ELX-02. Cellular motility, quantified by the migration index (MI), was calculated as the percentage of the microscopic field consumed by motility tracks.[36] Prior to treatment with ELX-02, RDEB1 and RDEB2 fibroblasts exhibited increased motility, with MIs of 43.68 and 39.80, respectively, in comparison to the MIs of 28.1 observed for NHFs (Figure 4B). In contrast, ELX-02-treated fibroblasts reversed their motility with MIs of 27.12 and 27.31, respectively, similar to NHFs. ELX-02 did not affect the motility of NHFs (data not shown). These results indicate that ELX-02-induced C7 is functional and can reverse the hypermotility of RDEB fibroblasts.

ELX-02 reverses the hypermotility and the poor substratum attachment characteristic of JEB keratinocytes

It has been shown that JEB cells display abnormal cellular phenotypes, including hypermotility and reduced cell adhesion.[37] To test the functionality of ELX-02-induced laminin 332, we conducted a keratinocyte migration assay and calculated MIs to assess the motility of both treated and untreated primary JEB keratinocytes. Figure 4C shows representative microscopic fields of NKCs and JEB1 and JEB2 keratinocytes untreated or treated with ELX-02. Prior to treatment with ELX-02, JEB1 and JEB2 keratinocytes exhibited increased motility, with MIs of 44.38 and 45.85, respectively, in comparison to the MI of 25.58 observed for NKC (Figure 4D). In contrast, ELX-02-treated keratinocytes showed normalized cellular motility with MIs of

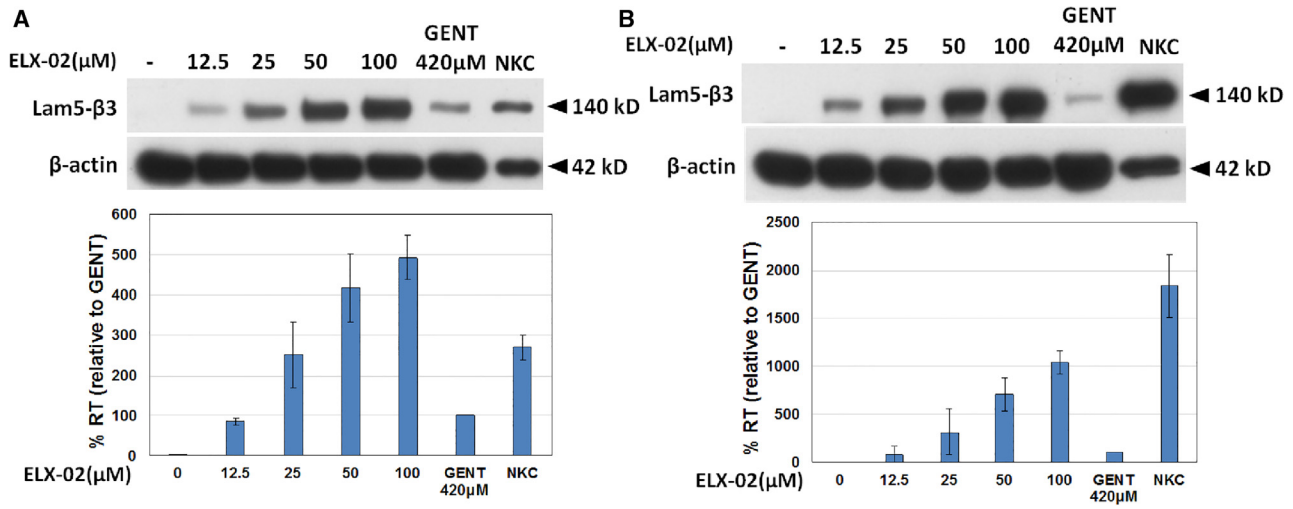

Figure 3. ELX-02 mediated dose-dependent induction of full-length laminin β3 in JEB keratinocytes

JEB1 (A) and JEB2 (B) primary keratinocytes were treated with increasing concentrations of ELX-02 and gentamicin (GENT) as indicated, for 48 h. Cell lysates were prepared and then subjected to 4%–12% SDS-PAGE, followed by immunoblot analysis with a monoclonal anti-laminin β3 antibody or an anti-β-actin (loading control) antibody. Note that ELX-02 induced full-length laminin β3 production in a dose-dependent manner in both JEB keratinocytes. The ImageJ analysis of laminin β3 expression normalized with β-actin is shown below the respective blots. The results are displayed as a percentage of the level of laminin β3 obtained from cells treated with GENT (100%). We performed three independent experiments, and similar results were obtained (see Figure S7). Error bars, SE of three different experiments.

25.03 and 24.51, respectively, similar to NKCs. Treatment with ELX-02 did not affect the motility of NKC (data not shown).

We next determined whether ELX-02-induced laminin 332 can improve the defective cell-substratum adhesion in JEB cells. JEB cells were subjected to a well-established kinetic cell-detachment assay with or without treatment with ELX-02. After the addition of trypsin, cells were detached from the substratum and quantified after 5 min. Prior to ELX-02 treatment, JEB cells (JEB 1 and JEB2) exhibited poor cell-substratum adhesion, with over 75% of JEB cells detaching within 5 min, while only 10% of NKCs detached (Figure 5). Conversely, following treatment with 75 μM ELX-02, ELX-02-induced laminin 332 in the treated JEB cells enhanced their cell-matrix adhesion strength to a degree similar to that seen in NKC. Treatment with ELX-02 did not affect the adhesion of NKC (data not shown). These results indicate that ELX-02-induced laminin 332 is capable of reversing the hypermotility and correcting poor cell-substratum adhesion that are characteristic of JEB cells.

ELX-02-induced C7 or laminin 332 localizes to the DEJ of RDEB and JEB skin equivalents

After demonstrating that ELX-02 restores the production of full-length C7 and laminin 332 and corrects abnormal phenotypes in RDEB and JEB cells, we sought to determine whether ELX-02-induced-C7 or laminin 332 incorporates into the DEJ. To test this, we established *in vitro* three-dimensional organotypic skin equivalents (SEs). SEs were constructed with primary RDEB or JEB keratinocytes untreated or treated with either ELX-02 or gentamicin and combined with C7-null RDEB fibroblasts with nonsense mutations. Up to 2 weeks after the SEs were established in culture, they were subjected to immunofluorescence staining using a polyclonal antibody to C7 or a polyclonal anti-laminin 332(β) antibody that recognizes the β3 chain. SEs generated from untreated RDEB or JEB keratinocytes demonstrated an absence of C7 or laminin 332 at the DEJ, as expected (Figures 6A and 6B). Furthermore, while SEs generated from RDEB or JEB keratinocytes treated with gentamicin or ELX-02 both demonstrated increased staining of C7 or laminin 332 at the DEJ, ELX-02-treated SEs demonstrated notably higher levels of staining. As expected, SEs generated from NKCs showed a robust linear expression of C7 or laminin 332 at the DEJ. Quantitative analysis using ImageJ revealed that the amount of C7 deposited at the DEJ in SEs composed of ELX-02 or gentamicin-treated RDEB keratinocytes was approximately 85.8% and 46.4%, respectively, compared to the level of C7 produced in SEs composed of NKCs (Figure 6C). In addition, SEs composed of ELX-02-treated RDEB keratinocytes produced approximately twice the amount of C7 at the DEJ compared to SEs treated with gentamicin. For the SEs generated from JEB cells, the levels of laminin 332 detected at the DEJ from either ELX-02 or gentamicin-treated cells were 150.5% or 64.7% of the levels of laminin 332 produced from SEs composed of NKCs (Figure 6D). Therefore, we conclude that ELX-02-induced C7 or laminin 332 is able to incorporate into its proper location at the DEJ *in vitro*.

DISCUSSION

In this study, we assessed the efficacy of ELX-02 in inducing read-through of PTCs in both RDEB and JEB. Our results indicate that ELX-02 is capable of inducing C7 production in primary RDEB fibroblasts and keratinocytes, as well as laminin 332 in primary JEB keratinocytes. This effect is dose-dependent, and the potency increases with prolonged dosing. It appears that ELX-02 is more effective

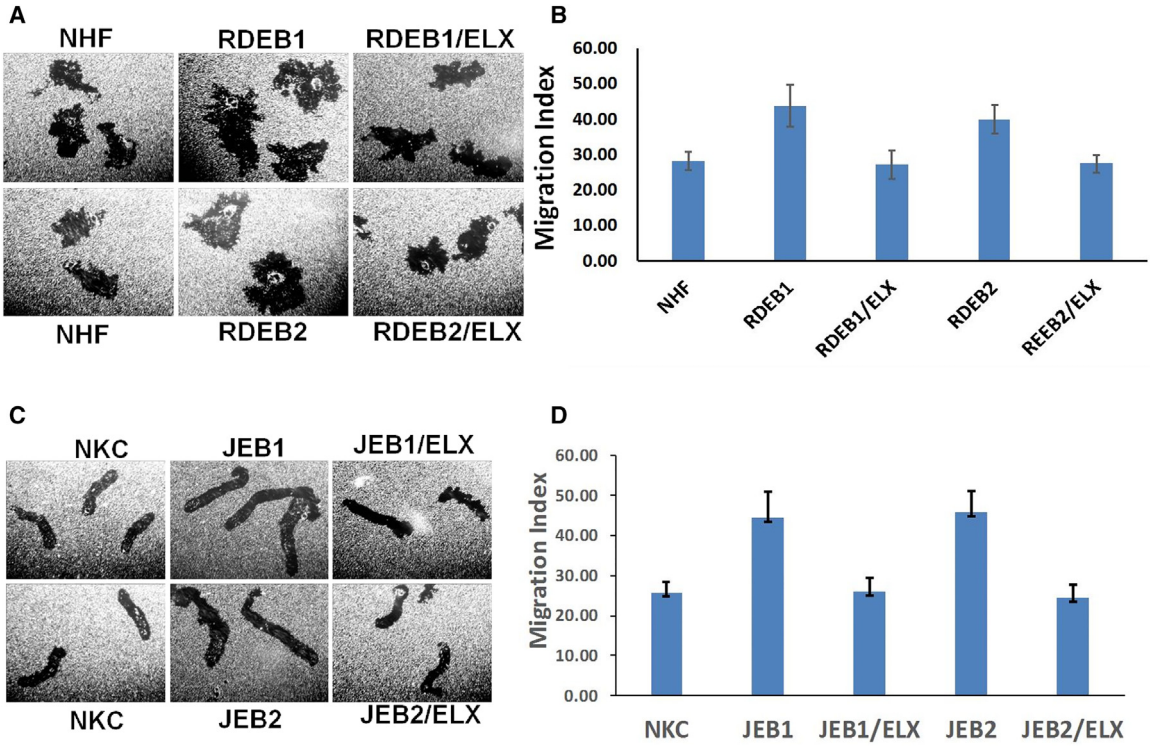

Figure 4. ELX-02 reversed RDEB fibroblast and JEB keratinocyte hypermotility

RDEB1/RDEB2 fibroblasts (A and B) and JEB1/JEB2 keratinocytes (C and D) were either untreated or treated with 100 μM of ELX-02 as indicated for 48 h and then subjected to a colloidal gold salt migration assay using collagen I as a matrix. (A and C) At left are representative fields photographed at 40× under dark-field optics. (B and D) At right are computer-generated MIs. The MI is the percentage of the total field area occupied by migration tracks. Error bars, SE of three different experiments. Note that both untreated RDEB1/RDEB2 fibroblasts and JEB1/JEB2 keratinocytes showed hypermotility in comparison with NHFs and NKCs, respectively. In contrast, the presence of ELX-02 normalized characteristic RDEB and JEB cell hypermotility.

than gentamicin in promoting PTC readthrough, resulting in greater production of C7 and laminin 332 in both RDEB and JEB cells, respectively. The functionality of C7 induced by ELX-02 in RDEB fibroblasts and laminin 332 induced in JEB keratinocytes is evident, as demonstrated by the reversal of characteristic hypermotility in RDEB and JEB cells and correcting the defective cell-substratum adhesion in JEB cells. Lastly, C7 and laminin 332 produced by ELX-02-treated RDEB and JEB keratinocytes incorporated into the DEJ in SE models.

Studies have postulated that the relative readthrough ability of a PTC mutation is influenced by the type of stop codon (UGA>UAG>UAA) and the nucleotides immediately downstream (C>U>G>A). For example, the stop codon UGA, when followed by a C, is most susceptible to aminoglycoside-mediated readthrough.[38,39] In a previous study, we did not find any correlation between the readthrough ability of gentamicin and PTC mutations in 22 cases of RDEB nonsense mutations. This observation was consistent across various types of stop codons, their contexts, and their proximity to exon-intron boundaries.[19] Consistent with our previous observation, the cells used in the study herein demonstrated a robust response to treatment with ELX-02 irrespective of the mutation type and contents. However, it is important to note that the number of mutations we tested here is limited, and more experiments need to be conducted with a variety of mutations to determine whether ELX-02-induced PTC readthrough is mutation specific. Of note, the JEB2 cells utilized in this study are heterozygous for the R635X/R42X mutation, with R635X being a hotspot nonsense mutation found in up to 84% of patients with a mutated *LAMB3* gene.[10,11] JEB2 cells demonstrated a strong response to ELX-02, resulting in laminin 332 levels up to 10.4 times higher than those induced by gentamicin, and reaching 56.6% of the levels observed in NKCs. These results indicate that ELX-02 will likely have a more robust treatment response in JEB patients than gentamicin given the large proportion of patients harboring this mutation.

The goal of an efficacious RDEB treatment is to produce a sufficient level of C7 that properly localizes to the DEJ and provides strong epidermal-dermal adherence. It is known that C7 is produced by both epidermal keratinocytes and dermal fibroblasts. Multiple lines of evidence suggest that reaching 35% of the physiologic amount of C7 is required to correct the RDEB phenotype and prevent skin blistering.[40] Heterozygous carriers of a *COL7A1* null mutation who have 50% of the normal complement of C7 and AFs are phenotypically normal, with no skin fragility or mechanobullous disease, unlike their

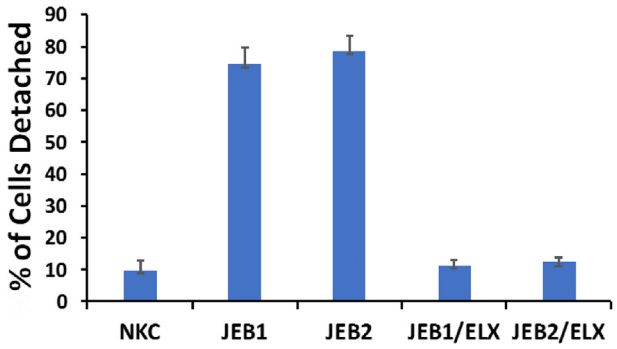

Figure 5. ELX-02 reversed the poor substratum attachment of JEB cells

JEB1 and JEB2 keratinocytes were treated with 75 μM ELX-02 for 48 h. All cells were simultaneously trypsinized, and the number of cells detached after 5 min was determined and expressed as a percentage of the total number of cells for each patient's cells. After 5 min, about 75% of JEB cells were detached, while NKCs and ELX-02-treated JEB1 and JEB2 keratinocytes exhibited less than 20% detachment. Each value is the average of triplicates from three independent experiments.

homozygous family members who suffer from RDEB.[41,42] In one study, the analysis of C7 quantity in the skin of RDEB patients indicated that their AFs were reduced by at least 77% compared to normal skin levels.[43] Another study observed that in RDEB-like C7 knockout mice, restoration of C7 to 35% of normal was sufficient for reasonable epidermal-dermal adherence against shear forces, but below this level, adherence was compromised.[40] In the present study, we found that levels of ELX-02-induced C7 in primary RDEB fibroblasts from two RDEB patients exceeded the levels induced by gentamicin and surpassed the target goal of 35% required for achieving strong epidermal-dermal adherence. Additionally, the C7 produced was able to reduce the hypermotility of RDEB fibroblasts, demonstrating that there is a sufficient amount of C7 to correct the RDEB phenotype. However, we found that levels of ELX-02-induced C7 in RDEB keratinocytes are less robust than that seen in RDEB fibroblasts from the same two patients. The reason why ELX-02 elicited a stronger response in RDEB fibroblasts compared to RDEB keratinocytes with the same mutations remains unknown. Nonetheless, previous therapeutic interventions using gene-corrected fibroblasts alone have shown enhanced dermal-epidermal adherence and clinical improvement in both pre-clinical and clinical trials.[44,45] Hence, an adequate ELX-02 induced PTC readthrough from fibroblasts alone might suffice to ameliorate the RDEB phenotype.

Similar to RDEB, the primary objective of JEB therapy is to achieve adequate levels of laminin 332 that will correctly localize to the DEJ, ensuring sufficient adherence between the dermis and epidermis. Unlike RDEB, which necessitates approximately 35% of the physiological amount of C7 to correct its phenotype, the minimum quantity of laminin 332 required to correct the JEB phenotype is not well defined. One study analyzing skin biopsies from a JEB patient with spontaneous *LAMA3* PTC readthrough noted that the patient continued to experience clinical improvement despite the patient's keratinocytes secreting significantly less laminin α3 than healthy controls in cell culture.[46] This suggests that the amount of laminin 332 secreted by keratinocytes required to provide adequate dermal-epidermal adherence at the DEJ may be significantly less than that produced by normal keratinocytes. In the present study, we found that ELX-02-induced laminin β3 was 183.3% and 56.6% of that seen in NKCs for JEB1 and JEB2, respectively, in cell cultures. The notably elevated level of laminin β3 expression in JEB1 keratinocytes compared to NKCs was consistent with a 150% increase in laminin 332 relative to normal keratinocytes in SE models constructed using JEB1 keratinocytes.

ELX-02 works by inducing PTC readthrough. In the presence of a nonsense mutation, the tRNA mechanism recognizes a PTC along the mRNA, leading to the production of a truncated and non-functional protein.[18] Treatments that promote PTC readthrough, such as ELX-02, function by decreasing translation-termination efficiency.[34] This results in the insertion of an amino acid at the aberrant stop codon site in eukaryotic mRNA. Therefore, translation continues, and a full-length protein is produced. Given the mechanism of PTC readthrough, there is a possibility that the resulting protein product could have altered functionality because of an incorrectly inserted amino acid in place of the stop codon. In fact, a study found that depending upon the severity of phenotype, 5%–10% of RDEB patients had missense mutations caused by the incorrect coding of a single amino acid in the *COL7A1* gene.[47] This study provides evidence that the protein products resulting from ELX-02-induced readthrough of nonsense mutations retain their functionality *in vitro*. The observed improvement in motility for both RDEB fibroblasts and JEB keratinocytes after ELX-02 treatment suggests that the C7 and laminin 332 produced effectively restores certain elements of fibroblast and keratinocyte functionality, leading to the correction of both RDEB and JEB abnormal cellular phenotypes. Additionally, ELX-02 treatment stimulated the production of laminin 332, which effectively corrected the defective cell-substratum adhesion in JEB cells. Finally, the localization of C7 and laminin 332 to the DEJ in RDEB and JEB SEs, respectively, suggests that ELX-02 induces proteins that home to the correct location. It is also important to note that increased ELX-02-induced C7 and laminin 332 expression may occur via increased mRNA expression, but real-time qPCR did not demonstrate increased mRNA expression in RDEB1 fibroblasts treated with ELX-02 (Figure S5).

Various other treatment options have been proposed for JEB and RDEB alike. Treatments suggested for JEB have included protein replacement therapy,[12] stem cell therapy,[11,15] and bone marrow transplantation.[14] Treatments under investigation for RDEB include protein replacement therapy by giving RDEB patients topical, intradermal, or intravenous recombinant human C7, cell therapy with bone marrow stem cells, allogeneic dermal fibroblast or allogeneic ABCB5^{+} mesenchymal stromal cells, transplantation of gene-corrected keratinocyte autografts, and localized therapy with topical HSV-1-based gene therapy.[7,48] However, none of the aforementioned therapies are consistently efficacious. Additionally, they are often difficult to implement and require the patient to endure frequent or

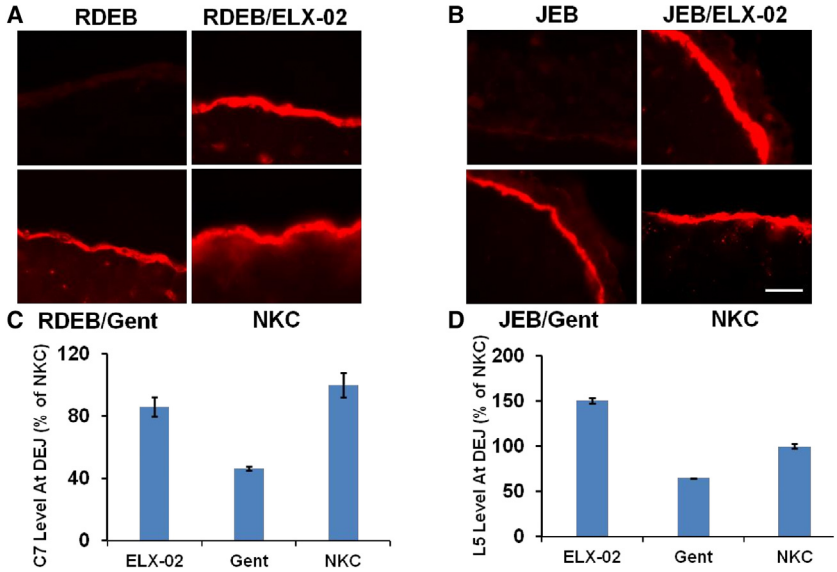

Figure 6. ELX-02-induced C7 or laminin 332 incorporated into the DEJ of *in vitro* SEs

Cryosections from 1-week SEs were subjected to immunofluorescent labeling using a polyclonal anti-C7 antibody (A) or anti-laminin 332 (β3) antibody (B). RDEB or JEB are SEs composed of RDEB keratinocytes or JEB keratinocytes combined with RDEB fibroblasts. RDEB/ELX-02, JEB/ELX-02, RDEB/Gent, and JEB/Gent are SEs composed of RDEB fibroblasts combined with RDEB or JEB keratinocytes treated with ELX-02 or gentamicin (400 μg/mL) before seeding and after plating onto dermal equivalents. NKCs are SEs composed of NHFs combined with NKCs. Intensity of C7 (C) or laminin 332 (D) at the DEJ of each specimen was measured by computer-assisted ImageJ software and compared to the intensity of type VII collagen (C7) or laminin 332 (L5) in SEs derived from NKC. Values represent the intensity of C7 or laminin 332 in the DEJ of the SEs expressed as a percentage of the average intensity obtained from NKC (set as 100%). Data represent the mean ± SE. Scale bars: 50 μm.

invasive treatment. While some of these treatments have demonstrated promising results, they are often limited by cost. The need for effective treatment for JEB is particularly urgent given the early mortality and severe morbidity associated with JEB. A compound such as ELX-02 can be produced in large quantities, does not expose patients to live cells or viral vectors, and does not require immunosuppression or invasive surgery. Additionally, we envision that ELX-02 may be delivered topically, subcutaneously, or intravenously, but more clinical testing is required to elucidate the best method of administration. The side effect profile of ELX-02 is also favorable, with only 27.5% of patients experiencing mild reactions at the injection site, compared to a 10% rate in those receiving a placebo.[28] Considering the results of the present study, ELX-02 is a promising treatment option for both RDEB and JEB.

In conclusion, this study demonstrates that ELX-02 is capable of inducing PTC readthrough and restoring functional C7 and laminin 332 in RDEB and JEB. Therefore, our study provides the proof of concept for using ELX-02 to suppress PTCs and induce C7 and laminin 332 expression in RDEB and JEB patients with nonsense mutations. The utility of ELX-02 for treating nonsense mutations associated with RDEB and JEB needs to be evaluated further in clinical trials. In addition, aminoglycoside derivatives that have higher readthrough activity and reduced toxicity when compared to gentamicin, such as ELX-02, should be of considerable clinical interest since about 25% of RDEB patients and 85% of JEB patients harbor nonsense mutations. Lastly, ELX-02-mediated PTC readthrough therapy may also be applied to other inherited skin diseases caused by nonsense mutations.

MATERIALS AND METHODS

Cell cultures

Primary dermal fibroblasts and epidermal keratinocytes from two RDEB patients, RDEB1 homozygous for R578X mutations and RDEB2 heterozygous for R163X and R1683X mutations, were previously established from the patients' skin biopsies[22] and cultured in DMEM/Ham's F12 (1:1) supplemented with 10% fetal bovine serum for primary RDEB fibroblasts and in EpiLife media supplemented with human keratinocyte growth supplement (HKG) (Thermo Fisher Scientific, Waltham, MA) for primary RDEB keratinocytes.[49] Primary JEB keratinocytes from two JEB patients, JEB1 heterozygous for C325X/c.629-12T>A mutations and JEB2 heterozygous for R42X and R635X mutations, were previously established from patients' skin biopsies and cultured in EpiLife media supplemented with HKGs (Thermo Fisher Scientific).[20] Primary human keratinocytes were purchased from Thermo Fisher Scientific. Normal human fibroblasts from neonatal foreskin were initiated into culture as described previously.[36] Primary fibroblasts were passaged as they reached confluence, and all experiments were performed on cells between passages 4 and 6.

Drug treatment and immunoblot analysis

In experiments where ELX-02 or gentamicin was used to induce PTC readthrough, RDEB keratinocytes or fibroblasts or JEB keratinocytes at 70%–80% confluency were exposed to ELX-02 (12.5–200 μM; Eloxx Therapeutics, Watertown, MA) or gentamicin (420–840 μM, Sigma, St. Louis, MO) for 48 h.

To determine the cellular expression of C7 or laminin β3 protein, cellular extracts were prepared 48 h after incubating with the above drugs as described and subjected to 4%–15% SDS-PAGE (Bio-Rad, Hercules, CA). Proteins were then electrotransferred onto a nitrocellulose membrane. The presence of C7 was detected with polyclonal antibodies to the NC1 domain of C7, followed by a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) and enhanced chemiluminescence detection reagent (GE Healthcare, Buckinghamshire, UK). The presence of laminin β3 monomer was detected with a monoclonal anti-laminin β3 antibody (Anti-Kalinin B1, clone 17, BD Biosciences, San Diego, CA).

To determine the cellular expression of C7 after multiple doses of ELX-02, RDEB fibroblasts and RDEB keratinocytes were plated at a density of 300,000 cells per well of 6-well plates. On the following day, media were replaced with fresh media, and cells were untreated or treated with 50 μM of ELX-02 daily for up to 5 days. Cells were lysed on various days after treatment and subjected to immunoblot analysis using a polyclonal antibody to C7 and a monoclonal antibody to β-actin (loading control).

Cell migration assay

Cell migration was assessed as described by Woodley et al.[50] RDEB fibroblasts or JEB keratinocytes were plated at a density of 300,000 cells per well of a 6-well plate. After 24 h, cells were untreated or treated with ELX-02 for 24 h. Media and compounds were then supplemented with indicated doses of compounds. After 48 h of treatment, cells were sub-cultured and subjected to cell migration assay. Colloidal gold salts were immobilized on coverslips and covered with type I collagen (15 mg/mL). Fibroblast or keratinocyte cultures were suspended, plated on the coverslips, and allowed to migrate for 16–20 h. The cells were fixed in 0.1% formaldehyde in PBS and examined under dark-field optics with a video camera attached to a computer equipped with a frame grabber. The computer analyzed 15–20 non-overlapping fields in each experimental condition with NIH ImageJ 1.6 and determined the percentage area of each field consumed by cell migration tracks to establish the MI.

Cell detachment assay

To determine the degree of ELX-02-induced cellular adherence, a trypsin-based detachment assay was employed.[10] Briefly, NKCs and primary JEB keratinocytes were seeded on 12-well tissue culture plates at a density of 2×10^4 cells per well. Twenty-four hours after seeding, the medium was changed to one containing ELX-02. After 48 h, 250 μL trypsin/EDTA was added to each well, and any detached cells were removed and counted after 5 min. An additional 250 μL trypsin/EDTA was added, and all remaining cells were allowed to detach and were subsequently counted. The percentages of cells detached were obtained, and the averages and SDs from three independent wells for each condition/cell line were calculated.

Establishment of *in vitro* organotypic SEs and immunofluorescence microscopy

Establishment of an *in vitro* skin co-culture model was performed as previously described.[10] Briefly, DMEM media (Corning, Discovery Labware, Bedford, MA) containing human RDEB fibroblasts (1×10^6 cells/mL) was mixed with rat tail collagen 1 solution (2.5 mg collagen/mL) (Corning) and 10× DMEM (Corning, Discovery Labware). This solution was neutralized with sodium bicarbonate, 1 mL of this solution was distributed into each 12-well insert (ThinCerts 12-well, 3 μM pore size; Greiner Bio-One, Monroe, NC), and the gels were allowed to polymerize. The fibroblast-infused collagen gel was then submerged in serum-free DMEM and incubated for 24 h. DMEM media was removed, and each dermal equivalent was covered with 50 μL of 50 μg/mL fibronectin (Sigma) in ultrapure water solution and allowed to incubate for 30 min. In the meantime, RDEB and JEB keratinocytes (untreated or pretreated with ELX-02 or gentamicin) were re-suspended in EpiLife media supplemented with HKGs + 5% fetal calf serum (FCS; Thermo Fisher Scientific) at a concentration of 1×10^6 cells/mL. Cells in this solution were seeded onto the fibronectin over each gel and incubated for 45 min to allow the cells to adhere. Afterward, the gels were submerged in EpiLife media supplemented with HKGs + 5% FCS and cultured for up to 10 days with descending FCS concentrations (5%, 2%, and 0% FCS) every 2–3 days that a media change occurred, with ELX-02 or gentamicin supplementation where appropriate. Between days 7 and 10 after keratinocyte seeding, SEs were extracted and placed on nitrocellulose strips after being soaked in PBS. The nitrocellulose-bound SEs were soaked in 50% sucrose solution for 90 min and then slow-frozen on a metal plate over dry ice. Frozen SEs were mounted in optical cutting temperature (OCT) and frozen. Five-micrometer-thick sections from the OCT-embedded SEs were cut using a cryostat, fixed for 5 min in cold acetone, and air dried. Immunolabeling of SEs was performed using standard immunofluorescence methods, as described previously.[10] SE sections were labeled with either a polyclonal antibody against C7 or a polyclonal anti-laminin 332(β) chain antibody, followed by a CY3-conjugated goat anti-rabbit IgG (1:1,000). Representative photographs from stained sections were taken using a Zeiss Axioplan fluorescence microscope equipped with a Zeiss Axiocam MRM digital camera system (Carl Zeiss, Oberkochen, Germany). All images were photographed using the same camera and at identical exposure times. Mean fluorescence intensity at the DEJ was calculated for each sample using ImageJ (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/), as described previously.[22]

Cell viability

For the ELX-02 cytotoxicity assay, RDEB fibroblasts were plated at a density of 20,000 cells per well of a 96-well plate. RDEB keratinocytes were plated at a density of 25,000 cells per well. JEB keratinocytes were plated at a density of 25,000 cells per well of a type I collagen-coated 96-well plate (required for cell attachment). At 24 h, cells were untreated or treated with escalating doses of ELX-02 for 24 h. After another 24 h, media were replaced with fresh media supplemented with indicated doses of ELX-02. Plates were allowed to incubate for 48 h. A freshly prepared solution of 4 mg 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]- 2H-tetrazolium hydroxide (XTT, Visalia, CA) in 4 mL of culture medium was mixed with 10 μL phenazine methosulfate (PMS; Sigma) solution (3 mg PMS in 1 mL PBS), and 25 μL of the combined XTT/PMS solution was directly added to each 100-μL cell culture.[10] Cultures were incubated for 4 h at 37°C, and absorbance was read at 450 nm.

Real-time qPCR

RDEB1 fibroblasts were grown until confluent and treated with ELX-02 (200 μM) or gentamicin (840 μM) for 24 h. Total RNAs were extracted using the Aurum total RNA kit as recommended by the manufacturer (Bio-Rad). RNA was quantified with a Nanodrop apparatus, and its quality was analyzed on an agarose gel. To amplify the *COL71A* mRNA, real-time qPCR was carried out using the following primers: forward (5′-GTTGGAGAGAAAGGTGACGAGG-3′) and reverse (5′-TGGTCTCCCTTTTCACCCACAG-3′) (product length

of 119 bp). β-Actin was also amplified using the following primers: forward (5′-CACCATTGGCAATGAGCGGTTC-3′) and reverse (5′-AGGTCTTTGCGGATGTCCACGT-3′) (product length of 135 bp). Real-time qPCR assays were then performed with LightCycler technology (Roche Mannheim, Mannheim, Germany). Experiments were carried out in triplicate, with the total mRNA from duplicate cell cultures.

DATA AND CODE AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the paper and its supplemental information.

ACKNOWLEDGMENTS

This work was supported by funding from Eloxx Pharmaceuticals.

AUTHOR CONTRIBUTIONS

Conceptualization: M.C., V.B., and S.A. Methodology: M.C. Validation: B.L., Y.H., X.T., K.Z., and M.C. Investigation: B.L., Y.H., X.T., K.Z., S.A., and M.C. Writing – original draft preparation: B.L., L.B., and M.C. Writing – review & editing: B.L., V.B., and M.C. Project administration: M.C. Supervision: M.C. Funding acquisition: M.C. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

V.B. is a former employee and current shareholder of Eloxx Pharmaceuticals, which owns patents related to this paper. S.A. is a former employee of Eloxx Pharmaceuticals.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102334.

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