Project Title: A Pilot Study of the Restoration of Full-Length Type VII Collagen in RDEB Patients with Nonsense Mutations After Topical, Intradermal and Intravenous Gentamicin Treatment

HSIRB Title: Gentamicin Therapy for Recessive Dystrophic Epidermolysis Bullosa Patients With Nonsense Mutations

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Abstract:

Recessive dystrophic epidermolysis bullosa (RDEB) is an incurable, devastating, inherited skin disease caused by mutations in the *COL7A1* gene that encodes for type VII collagen (C7), the major component of anchoring fibrils (AFs), structures that mediate epidermal-dermal adherence. Thirty percent of RDEB patients have nonsense mutations. We recently demonstrated in 5 such patients that intradermal and topical gentamicin induced “read-through” of their nonsense mutations and created robust and sustained new C7 and AFs at the dermal-epidermal junction (DEJ) of their skin and also improved wound closure and the quality of their skin. No untoward side effects occurred. Herein, we propose evaluating the safety and efficacy of intravenous gentamicin in these patients. We also propose optimizing the concentration and manner of delivery of topical gentamicin. The unambiguous milestones will be increased C7 and AFs in the patients’ DEJ, improved EB Disease Activity Scores, and absence of significant gentamicin side effects.

A. Background, Significance and Preliminary Data:

Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited, incurable, mechano-bullous disease characterized by blisters, erosions and scarring of the skin and mucosa.[1] RDEB is caused by mutations in the *COL7A1* gene that encodes type VII collagen (C7), a major component of anchoring fibrils (AFs), structures that hold the two main layers of skin together - the epidermis and the dermis.[2,3] Based largely on "pre-clinical" animal models, we and others envisioned several therapeutic strategies for RDEB. These include intradermal injections of allogeneic and RDEB gene-corrected dermal fibroblasts[4,5], topical and intradermal administration of C7 itself[6-8], intradermal injection of viral vectors expressing full-length C7 and intravenous C7 or fibroblasts that home to RDEB skin wounds[9-11], and transplantation of gene-corrected keratinocyte autografts.[12,13] Recently, proof-of-principle clinical trials have been conducted, including bone marrow/stem cell transplantation, intradermal injection of allogeneic fibroblasts and transplantation of gene-corrected keratinocyte autograft sheets onto RDEB skin wounds.[14,15] These strategies are logistically arduous and not consistently effective or safe. The mainstays of RDEB treatment currently are only palliative and supportive measures.

Over 800 distinct mutations (missense, frame-shift, insertion, deletion, and nonsense changes) have been identified in RDEB patients.[16,17] Our recent search of an RDEB patient registry data base shows that the prevalence of nonsense mutations in RDEB approaches 30% (161 out of 506 patients),[16,17] and these patients often have a more severe form of the disease.[37] Aminoglycoside antibiotics can read-through nonsense mutations and generate full-length, functional proteins in cystic fibrosis (CF), Duchennes' muscular dystrophy (DMD) and other genetic diseases with nonsense mutations[18,19] Gentamicin is an aminoglycoside antibiotic that has been used for infectious diseases for decades. It also is the most effective aminoglycoside for reading through nonsense mutations in CF and DMD and can be delivered topically or intravenously.[20-25] Using cultured skin cells from 4 RDEB patients with nonsense mutations, we recently published that aminoglycosides were able to induce PTC read-through in the *COL7A1* gene and produce full-length C7.[26] Importantly, aminoglycoside-induced C7 reversed the abnormal RDEB cell phenotype and incorporated into the DEJ of skin equivalents. In addition, we showed that aminoglycosides were capable of promoting PTC read-through and inducing full-length C7 in human 293 cells transfected with C7 expression constructs harboring 22 different known RDEB nonsense mutations.[26] With these exciting data in hand, we were fortunate to secure funding from Epidermolysis Bullous Research Partnership (EBRP) and translated these *in vitro* findings into patients. For this clinical trial, we recruited five patients with nonsense mutations from our 22 RDEB patient cohort[27]and treated them with topical and intradermal gentamicin (please see details of NCT02698735 on ClinicalTrials.gov). **Table I** summarizes the participants. The study was double-blinded and placebo-controlled at its onset. For the topical arm, gentamicin 0.1% ointment or the ointment vehicle were applied three times a day to two open erosion Test Sites of at least 2 x 2 cm in size for two weeks. For the intradermal arm, in the same patients, a sterile gentamicin solution (40 mgs/ml) or sterile saline placebo were injected intradermally into two skin Test Sites, remote from the topical Test Sites, on days 1 and 2. On each day 200 µl (8 mgs) were injected into each site for a total dose of 16 mgs. Follow-up visits were at weeks 4 and 12. The patients completed a weekly telephone questionnaire. They also kept a daily diary and photographed their skin test sites once a week. A number of safety parameters were also assessed including a complete blood count, blood urea nitrogen, creatinine, electrolytes, liver function tests, pure-tone audiometry, and serum ELISAs and indirect immunofluorescence for the possible development of anti-C7 autoantibodies. Briefly, the results of the safety parameters showed no untoward, gentamicin-induced altered audiometry or laboratory abnormalities. In addition, we did not detect any gentamicin-induced anti-C7 antibodies in the patients.

Patient ID	Allele 1 / Allele 2	C7 Level at DEJ by pAb	C7 Level at DEJ by mAb	Anchoring Fibrils by EM	
				Density	Morphology
1	**R578X** / V168GfsX12	2%	Absent	+	Very thin and wispy
2*	**R578X** / **R578X**	Absent	Absent	+	Short, rudimentary
3	IVS17-2delA/ **R2814X**	4.5%	Absent	+	Thin and wispy
4	**R236X** / IVS85-1G>A	11.6%	2%	+	Thin and wispy
5*	**R613X** / **R1683X**	<1%	Absent	+	Thin, mild arching
NHS	- / -	100%	100%	+++++	Thick, banded, arching, looping

Table I. Summary of *COL7A1* mutations, basal C7 levels and AFs in 5 RDEB patients.* indicate children; pAb, polyclonal antibody; mAb, monoclonal antibody.

All 4 patients trended towards clinical improvement in the gentamicin Test Sites with less new blister formation, rapid closure of existing erosions and improved skin durability compared to the placebo Test Sites. **Figure 1A** shows wound sites that received topical gentamicin or placebo from Patient #3. Topical gentamicin rapidly closed the Test Site wound and kept it closed for 12 weeks. Patients reported improved wound closure and skin quality. **Figure 1B and 1C** shows the patient's expression of C7 and AFs at her DEJ before and after topical or intradermal gentamicin. At Day 0, there was minimal or no C7 at the patient's DEJ. At the 4-week visit, there was minimal C7 at skin sites that received the topical and intradermal placebos. In contrast, there was abundant C7 expression at the DEJ of the patient's skin after topical or intradermal gentamicin at week 4.

Surprisingly, gentamicin-induced C7 was stably retained at the DEJ at 12 weeks after initial application. Immuno-EM with a monoclonal antibody to C7 revealed marked labeling of new AFs (arrows) in the patient's skin 4 weeks after topical gentamicin. At 12 weeks, there were plentiful, well-labeled AFs in both the topical and intradermal gentamicin sites. In contrast, there were neither labeling nor visible AFs in the placebo treated sites. **Table II** summarizes the immunofluorescence (IF) results of the study. The numbers are the percentage of C7 expression compared with normal skin. All 5 patients responded to topical and intradermal gentamicin with maximal C7 levels restored for each patient ranging from 19% to 166% of that of normal human skin. The study of our 5th patient is not yet completed. In a preliminary pilot study with one patient, we noted that the topical application of gentamicin to unwounded skin resulted in new C7 in Patient #4 to 65% of that of normal skin (E2 in **Figure 2**). This raises the possibility that perhaps topical gentamicin can also penetrate intact skin.

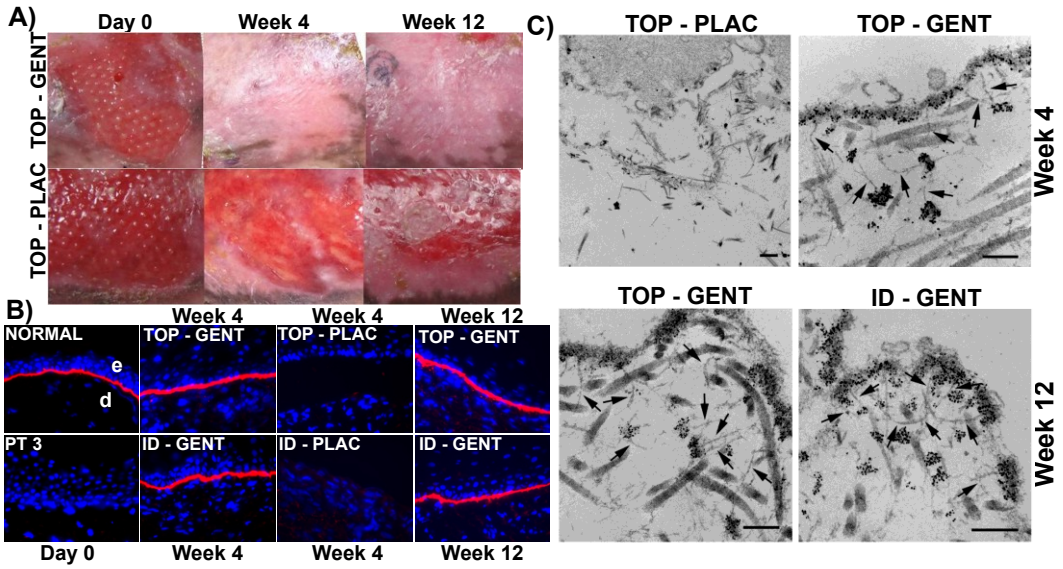

Figure 1. Gentamicin improved wound closure and restored C7 and AFs in RDEB patient. (A) Shown are patient #3's wound sites treated with topical gentamicin (TOP-GENT) or placebo (TOP-PLAC) at day 0, and weeks 4 and 12. **(B)** Cryosections from day 0, weeks 4 and 12 were subjected to immunofluorescence labeling with a monoclonal antibody against C7, after treatment with topical gentamicin (TOP-GENT), topical placebo vehicle (TOP-PLAC), intradermally injected gentamicin (ID-GENT) or intradermally injected saline placebo (ID-PLAC). Note that topical or intradermal gentamicin induced strong C7 at the DEJ. **(C)** Skin sections were subjected to immuno-EM following incubation with a monoclonal antibody, NP185 to C7. Please note that in the placebo treated wounds (TOP-PLAC, top left panel), there was no labeling of the DEJ and no visible AFs. In contrast, skin biopsy samples from both topical (TOP-GENT) and intradermally injected gentamicin sites (ID-GENT) exhibited heavy gold labeling of the lamina densa and anchoring plaques. In addition, many well-labeled, wheat-stack shaped AFs are readily observed (arrows). Scale bars: 250 nm.

B: Innovation: Our clinical trial above showed that topical and intradermal gentamicin induced new C7 and new AFs. This has never been shown before. We now wish to determine if systemic intravenous gentamicin can improve multiple skin wounds simultaneously (including the esophagus) in the patients and perhaps prevent new blister formation. With this systemic administration and improvement in multiple wound sites, there may be improved patient clinical scores and improved patient quality of life. This type of systemic treatment was completed in CF and DMD patients,[22-25] but never before in RDEB patients. Secondly, in this proposal, we wish to determine if we can optimize topical gentamicin delivery into the patient's papillary dermis using a five-fold increase in the concentration of the drug or delivering the gentamicin with a micro-needle device.

Patient ID	Topical 1M		Topical 3M		ID 1M		ID 3M	
	Gent.	*Plac.*	*Gent.*	*Plac.*	*Gent.*	*Plac.*	*Gent.*	*Plac.*
1	N/A	0	**50**	0	**42**	0	**40**	0
2	**19**	0	**18**	**0**	**19**	**0**	**1**	**0**
3	**153**	0	**165**	**0**	**121**	**0**	**120**	**0**
4	**166**	**7**	**50**	**6**	**108**	**5**	**51**	**3**
5	**20**	0	NYD	NYD	NYD	NYD	NYD	NYD

Table II: Image J analysis of IF results of C7 levels at DEJ in comparison with normal human skin.

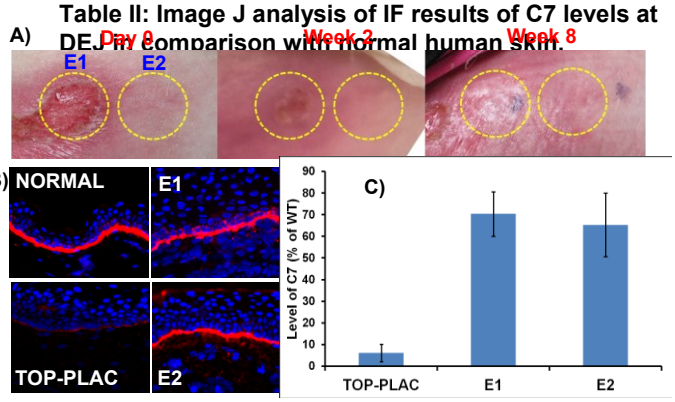

Figure 2. Gentamicin restored C7 in RDEB patient. (A) Shown are Patient #4's open (E1) and closed (E2) wound sites at day 0, and weeks 2 and 8 after topical gentamicin. **(B)** Cryosections from topical placebo and gentamicin at week 8 were subjected to IF with a polyclonal antibody against C7. **(C)** Image J analysis of IF results of C7 levels at DEJ compared to normal human skin.

C: Approach:

Aim 1. Evaluate the Safety and Efficacy of Short Term Intravenous Gentamicin for RDEB Patients.

Overview & Rationale: The rationale for short-term systemic intravenous gentamicin in these patients is outlined in the sections above, namely the drug could treat multiple skin sites simultaneously including the esophagus. This approach has proven safe and efficacious in other genetic diseases such as CF and DMD[22-25] Our

HYPOTHESIS is that intravenous gentamicin will, like topical and intradermal gentamicin, create new C7 and AFs at the patient's DEJ, improve multiple skin sites simultaneously, improve the patient's clinical disease and improve his or her quality of life. In accordance with the enclosed letters from two experienced pediatricians, we believe this therapy will be safe. Because C7 and AFs are incredibly stable, we further hypothesize that newly induced C7 and AFs will persist for many months in the patient's skin. Using dosing regimens of gentamicin that have proven to be safe, we will: **1)** determine the percentage increase of C7 expression in the patients' skin before and after treatment, **2)** determine how durable and sustainable the new C7 and AFs are in the patients' skin after treatment, **3)** determine if this treatment is safe in this patient population, **4)** determine if this treatment improves the patients clinically and improves their quality of life and **5)** generate data for a multi-centered Phase III study using a larger number of patients.

Patients/Intervention: We have already lined up 3 well-characterized, adult RDEB patients with nonsense mutations who wish to participate in this study. These patients had positive responses to topical and intradermal gentamicin in our clinical trial (**Table II**). The patients will receive intravenous gentamicin (7.5 mgs per kilogram), in 100 ml of 5% dextrose in saline, for 14 consecutive days, a dose proven to not cause ototoxicity or neprhotoxicity[28] and to be efficacious in inducing PTC read-through in DMD patients.[22] The first few infusions will be done in the Infusion Center of the Keck Hospital of USC. We will measure gentamicin trough levels (24 hours after infusion) and gentamicin peak levels (30 minutes after the infusion) during the second day and, if needed, adjust the gentamicin doses such that the peak level is between 20 and 40 µg/ml and the trough level <2 µg/ml. If no untoward side effects occur, the patients will then have their infusions in their home via a commercial infusion service.

Safety Evaluation: Patients will be consented, enrolled and evaluated on Day 0 and then evaluated on post-treatment days 30, 90, and 180. On Day 0, the patients will have a complete medical history, review of systems (ROS), physical examination, vital signs, and weight. They will also have baseline urine analysis, complete blood count (CBC), electrolytes, glucose, comprehensive metabolic panel (CMP), and calculated creatinine clearance. Pure tone audiometry and a calculated creatinine clearance will be done on Days 0, 7, 14 and 30 post-treatment. Stop criteria will be a decline of >15 dB on pure tone audiometry at 2 consecutive frequencies and a creatinine clearance <60ml/min.[28] Whenever there is new protein created in a patient, there is the possibility that the patient's immune system will raise autoantibodies against it. This did not occur in our clinical trial with topical and intradermal gentamicin, but it behooves us to evaluate this again with intravenous gentamicin and potentially more auto-antigenic protein created. Using indirect immunofluorescence and our anti-C7 ELISA, we will test the subject's serum for anti-C7 antibodies at each visit.

Efficacy: **A. Clinical Evaluations:** At baseline Day 0 and at each follow-up visit, (days 30, 90 and 180) the patients will complete a Quality of Life Assessment tool, a Pruritus Score tool, and a validated epidermolysis bullosa disease activity index (EBDASI)[29,30]. Patients will keep a daily diary using a self-ROS questionnaire. Patients will be telephoned weekly by a USC study member inquiring about any new signs, symptoms or ROS changes since the advent of the study. At baseline Day 0, we will identify four Test Sites at least 25 cm^2 in size to follow sequentially. Two will be from areas that at baseline have blisters or erosions and the other two from intact skin areas. The two Test Sites with active blisters and erosions will be traced using a transparency sheet and the areas of the lesions calculated on a digitizing tablet. Likewise, all of the Test Sites will have standardized photographs and the lesional areas calculated by computer-assisted morphometry, as previously published.[29-31] Within the context of their daily diary, patients will note the number of new blisters and erosions that occur in the Test Sites. A USC study member will telephone the patient each week and inquire about the Test Sites and ask the patient to estimate the percentage area of the Test Site consumed by erosions and blisters. Also, at each follow-up visit, three dermatologists will observe the four Test Sites and count the number of bullae and erosions in each. The intact Test Site areas will be scored at each follow-up visit using the following 3-point scale: 0 = no lesions; +1 = any active lesions; +2 = active lesions consuming over 50% of the Test Site area. Tracings to evaluate the areas of blisters or open erosions will be done, and standardized photographs paired with computer-assisted morphometry to calculate the areas of any lesions will be performed. **B. Evaluations of C7 and AF Expression:** At each post-treatment time point, 8 mm punch biopsies from the four Test Sites will be divided into three parts for **(i)** H&E histology and quantitation of epidermal-dermal adherence, **(ii)** the expression of C7 at the DEJ relative to that of normal human skin using anti-C7 antibodies to the NC1 and NC2 domains of C7 and NIH J Image software. One major "milestone" of the study will be the expression of NC2 since this would clearly indicate restoration of full-length C7 and (**III**) evaluation of newly created AFs by immuno-EM and AF enumeration by computer-assisted morphometry. Performing these evaluations out to 6 months will determine how durable the newly created C7 and AFs are. In our recent clinical trial outlined above, it was surprising that gentamicin-induced C7 remained at a similar level at 3 months post-treatment as that of 1 month for Patient #3 (Please see **Figure 1 and Table II**).

Aim 2: Evaluate the Safety and Efficacy of Higher Dose Topical Gentamicin for RDEB Patients.

Overview and Rationale: In our gentamicin clinical trial outlined earlier, there was significant variation in the production of new C7 and AFs, as shown in **Table II.** Patients #2 and #5 only restored C7 to 20% of normal skin in response to gentamicin. We only used one concentration of gentamicin, 0.1% ointment, and our *in vitro* studies with RDEB fibroblasts from these same two patients showed that the C7 response to gentamicin was dose-dependent.[26] In these patients, there could have been sub-optimal dosing or sub-optimal access of the gentamicin to the patient's dermal fibroblasts and basal keratinocytes. In this aim, we will use 0.5% gentamicin ointment rather than 0.1% and determine if the C7 response is more robust in these two patients. Based on our encouraging data shown in **Figure 2**, we will also treat intact skin and determine if higher dose gentamicin can prophylactically prevent frank skin blisters and erosions from forming.

Patients/Intervention: We will study two RDEB children tested in our previous trial for this study. In addition, we also have 2 other children and 2 other adult patients who have contacted us and wish to participate in this study (**Table III).** Two of these patients share the same mutations (R578X and R2814) as our current patients. We will select a total of four Test Sites (two with open wounds of at least 3 cm by 3 cm and two areas of intact skin nearby the Test Sites with blisters or erosions) and apply 0.5% gentamicin ointment two times a day under an occlusive dressing of the patients' choice for two weeks. This dose was used in combination with 15% paromomycin to ulcerative cutaneous leishmaniasis lesions once daily for 20 days in two publications without renal or ototoxicity detected.[32,33]

Patient ID	Allele 1 / Allele 2
6	**R578X**/G1907D
7	**R1933X**/G1907D
8*	**Y311X**/IVS106+11+26del16
9*	**R2814X**/G2132D

Table III. Four potential RDEB patients with nonsense mutations. *Children

Safety and Efficacy: We will essentially assess in these patients the safety and efficacy parameters outlined in Aim #1 at months 1, 3 and 6 after topical application. Potential renal toxicity and ototoxicity will be monitored by creatinine clearance and pure tone audiometry at day 0 and at each follow up visit.

Aim 3: Evaluate the Safety and Efficacy of Intradermal Administration of Gentamicin with a MR2 Micro-needle Roller Device.

Overview and Rationale: In our current clinical trial outlined above, there were excellent C7 responses to intradermal injections of gentamicin solution in several patients (**Table II**). Nevertheless, these injections were very localized with diffusion of the drug probably less than an inch from the single injection site. Recent evidence has shown that a MR2 microneedle roller device can readily deliver lidocaine into the lower epidermis and upper papillary dermis over a wide area of skin with good efficacy, safety and excellent patient tolerance.[34] This may be an opportunity to deliver intradermal gentamicin solution (40 mg/ml) to wide areas of skin in a simple manner that can be done in an outpatient office with minimal logistical issues and avoidance of systemic exposure to the drug. Advantages include the possibility of delivering in a "pulse " fashion significant levels of gentamicin to the papillary dermis over wide areas of skin with minimal discomfort and minimal systemic exposure of gentamicin. Avoiding systemic exposure would be valuable for RDEB patients who have ototoxicity or renal compromise, poor venous access or cannot tolerate intravenous infusions.

Patients & Interventions: We will enroll two new RDEB patients (either adults or children) shown in **Table III** in this "proof-of-principle" study. Skin Test Sites will be areas measuring at least 40 cm^2, and we will test one site of intact unwounded skin and one site of skin with lesions (blisters or erosions). The areas will be cleansed with 4% Hibiclens and then the MR2 Microneedle Roller Device passed over the area in two directions at 90 degrees. This device induces tiny micro-wounds in the skin to the depth of the papillary dermis. Gentamicin sterile solution (40 mg/ml) will then be liberally applied over the area and the area immediately occluded with a polyrurethane membrane (Opsite). Another equal sized Test Site of intact skin will be tested without prior intervention with the Microneedle Roller. The same gentamicin solution will be applied to the site and immediately occluded with Opsite. This will determine if the gentamicin solution can penetrate intact skin.

Safety and Efficacy: We will measure gentamicin in the serum of these patient 30 minutes after the microneedle delivery to determine if there is any systemic exposure when gentamicin is administered in this manner. Efficacy of the gentamicin administration will be assessed identically to those in Aim#1 by IF and immuno-EM analysis in conjunction with the patients keeping a daily diary of the treatment sites, weekly photographs of Test Sites, weekly staff interviews of the patients, and physician assessments at patient visits at months 1, 3 and 6 after administration of gentamicin. Potential renal toxicity and ototoxicity will be monitored by creatinine clearance and pure tone audiometry at day 0 and each follow up visit.

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