Project Title: A Pilot Study of the Effect of Topical and Intradermal Gentamicin on the Restoration of Full-Length Type VII Collagen in RDEB Patients Carrying Nonsense Mutations

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Abstract:

Recessive dystrophic epidermolysis bullosa (RDEB) is an incurable, devastating, inherited skin disease for which there is only supportive care. RDEB is due to mutations in *COL7A1* gene that encodes for type VII collagen (C7), the major component of anchoring fibrils (AFs) mediating epidermal-dermal adherence. Approximately 20% of *COL7A1* mutations are nonsense mutations leading to premature stop codons and a truncated C7 with diminished function. We demonstrated that aminoglycosides such as gentamicin readily induce PTC “read through” and produce biologically functional C7 in 22 reported *COL7A1* nonsense mutations. Importantly, aminoglycoside-induced C7 reversed the abnormal RDEB cell phenotype and incorporated into the dermal-epidermal junction. Herein, we propose the first clinical trial of gentamicin (topical and intradermal) in RDEB patients with nonsense mutations that we have fully characterized. The milestones include increased C7 and AFs at the patients’ dermal-epidermal junction, improved EB Disease Activity Score, and absence of significant gentamicin side effects.

Background and Significance:
Recessive Dystrophic Epidermolysis Bullosa (RDEB) is an inherited skin disease characterized by skin fragility blisters, erosions, and scarring.[1] RDEB is caused by mutations in the *COL7A1* gene that encodes type VII collagen (C7), the major component of anchoring fibrils (AFs).[2,3] Various therapeutic strategies have been envisioned for RDEB based on preclinical animal models: intradermal injection of allogeneic dermal fibroblasts or gene corrected RDEB fibroblasts,[4,5] intradermal injection of lentiviral vectors expressing C7,[6] intradermal injection or topical application of recombinant C7,[7-9] intravenous (IV) injection of C7 protein or fibroblasts,[10,11] and transplantation of gene-corrected keratinocyte autografts.[12,13] Recently, proof-of-principle clinical trials have been initiated, including bone marrow/stem cell transplantation and intradermal injection of allogeneic fibroblasts.[5,14] None of these therapies are consistently effective, and some have associated morbidity and mortality.

Over 700 distinct mutations have been identified in RDEB patients.[15]These mutations include missense, frame-shift, insertion, deletion, and nonsense changes. Between 10-25% of RDEB mutations are nonsense mutations that create a premature termination codon (PTC) resulting in a truncated, non-functional protein. RDEB patients with nonsense mutations often have severe disease. Aminoglycoside antibiotics such as gentamicin have emerged as vanguard agents for the treatment of human genetic disorders due to their ability to suppress PTC mutations and restore synthesis of a full-length protein. They have been used successfully in a number of genetic disorders, such as cystic fibrosis (CF), Duchenne's muscular dystrophy (DMD), and others.[16-18] Gentamicin, has been used in humans for many years and is the most effective aminoglycoside for inducing PTC read-through. Topical gentamicin to the nasal epithelium of CF patients restores their mutated receptor, CFTR.[19,20] Intravenous gentamicin has also improved disease in patients with CF and DMD.[21,22]

Using RDEB keratinocytes and fibroblasts harboring nonsense mutations, we showed that aminoglycosides (including gentamicin) induced PTC read-through and restored functional full length C7 that reversed the abnormal cell motility associated with RDEB **(Figure 1A to 1C**).[23] Using an *in vitro*, three-dimensional, skin equivalent model, we showed that C7 produced by aminoglycoside-treated RDEB cells incorporated into the dermal-epidermal junction (DEJ) of the skin equivalents (**Figure 1D**). We also generated 22 known RDEB nonsense mutations via site-directed mutagenesis and transfected these constructs into human 293 cells. Aminoglycoside treatment of these cells induced PTC read-through and induction of full-length C7 with all 22 RDEB nonsense mutations. This is the first study demonstrating that aminoglycosides can induce PTC read-through and restore functional C7 in RDEB caused by nonsense mutations. Our results suggest that aminoglycoside-mediated nonsense mutation suppression may provide a novel, non-invasive treatment for a sub-set of RDEB patients. The goal of this proposal is to determine if topical, intradermal, and intravenous gentamicin can suppress PTCs and induce full length, functional C7 in RDEB patients with nonsense mutations and to evaluate the safety, potential immune responses, efficacy, and duration of any beneficial effects. Our over-arching **HYPOTHESIS** is that the administration of gentamicin to RDEB patients will induce PTC read-through, create new

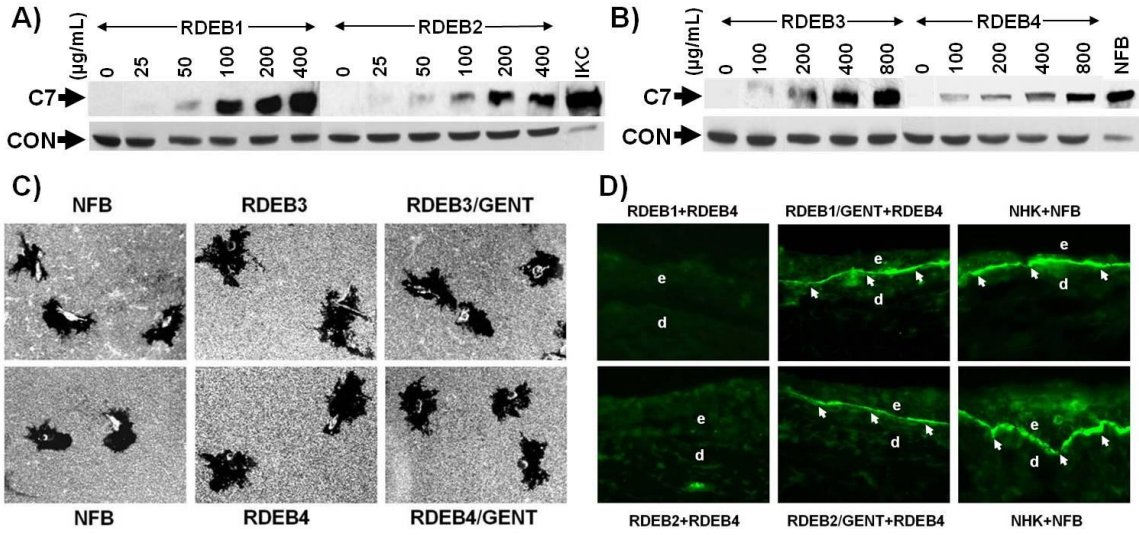

Figure 1. (A&B) Gentamicin mediated dose-dependent induction of full-length C7 in RDEB Cells. RDEB keratinocytes (A) or fibroblasts (B), were treated with increasing concentrations of gentamicin and cell lysates were prepared and then subjected to 4-12% SDS-PAGE, followed by immunoblot analysis with an antibody to C7 or anti-β-tubulin (loading control) antibody. **(C) Aminoglycoside-induced C7 reverses fibroblast hypermotility.** RDEB fibroblasts were either untreated or treated with gentamicin (400 μg/ml) for 48 hours and then subjected to a colloidal gold salt migration assay. Note that hypermotility of both RDEB fibroblasts were reversed after gentamicin treatment. **(D) Aminoglycoside-induced C7 incorporates into the DEJ of *in vitro* skin equivalents (SEs).** The cryosections were subjected to IF using anti-C7 antibody. Left panels are SEs composed of untreated RDEB keratinocytes combined with untreated RDEB fibroblasts. Middle panels are SEs composed of RDEB keratinocytes treated with gentamicin (400 μg/ml) combined with RDEB fibroblasts. Right panels are SEs composed of normal skin cells.

C7 and AFs at the DEJ, and improve the clinical status of these patients. We also hypothesize that clinical improvement will correlate with the level of gentamicin-induced PTC readthrough and C7 production. Finally, we hypothesize that the gentamicin–induced C7 will persist for several months and will not cause any adverse effects. Based on these hypotheses we propose two specific aims:

Aim: Evaluate the safety and efficacy of topical gentamicin for RDEB patients.

A. Identification of 5 RDEB patients with nonsense mutations for a pilot clinical trial.

We recently identified and characterized 22 *bona fide* RDEB patients clinically, genetically, pathologically, ultrastructurally and immunologically (Table 1).[24] All 22 RDEB patients had abnormal AFs with reduced density and significant scarring of their skin. We placed these patients' keratinocytes and fibroblasts into tissue culture allowing us to study their cellular phenotype and gene expression profiles. Five of 22 RDEB patients contain one

Patient ID	Allele 1 / Allele 2	Clinical Diagnosis	C7 Expression at DEJ	Anchoring Fibrils by EM	
				Density	Morphology
A	G2517KfsX3 / G2517KfsX3	RDEB-sev,gen	Reduced	+	Very thin and wispy
B	**R578X** / **R578X**	RDEB-sev,gen	Absent	+	Short, rudimentary
C	**R613X** / **R1683X**	RDEB-sev,gen	Reduced	+	Thin, mild arching
D	IVS17-2delA/ **R2814X**	RDEB-sev,gen	Reduced	+	Thin and wispy
E	**R236X** / IVS85-1G>A	RDEB-sev,gen	Reduced	+	Thin and wispy
F	**R578X** / G1907D	RDEB-I	Normal	+++	Non-banded, arching
NHS	- / -	Normal	Normal	+++++	Thick, banded, arching, looping

or two alleles of nonsense mutations (highlighted by bold in **Table 1**). We recently treated cultured fibroblasts of two patients (B and C) of these five patients with gentamicin.[23] The gentamicin treatment resulted in read-through of the nonsense mutations and generation of full-length C7 (See Figure 1B RDEB3 and RDEB4). In CF patients, there is a good correlation between the *in vitro* cell culture read-through levels and *in vivo* efficacy suggesting that *in vitro* studies should be done first to predict which patients are most likely to respond and benefit from aminoglycosides.[22] Therefore, we will treat the five gentamicin-responding RDEB patients with nonsense mutations identified in our recent study. We expect gentamicin treatment to be more effective in patients B and C who have two alleles with nonsense mutations than patients D to F who only have one nonsense mutated allele. In addition, one RDEB patient who has PTCs from frame shift mutations (A) will be treated to serve as a negative control patient since gentamicin is specific for nonsense mutation induced - PTCs. The Exclusion Criteria for these 6 RDEB patients will include pregnant women, breast feeding women, pre-existing auditory impairment, renal impairment, allergies to aminoglycosides, and use of aminoglyosides within the past 6 months. The acceptable renal parameters with be a normal BUN, normal plasma creatinine and a normal estimated creatinine clearance.

B. C7 and AF expression with topical and intradermal gentamicin treatment

i. ***Topical application of gentamicin to skin lesion:*** We will apply commercially available gentamicin 0.1% ointment three times a day under Tegaderm occlusion for two weeks to a target RDEB skin erosion. Likewise, we will apply the ointment control vehicle three times a day to a similarly sized RDEB erosion. This dosage schedule is based on the successful clinical trial in CF patients that resulted in clinical efficacy without side effects.[19] In our *in vitro* cell culture studies, we showed that a single dose of gentamicin (400 μg/ml) induced C7 expression at a level of 20-40% of that seen in normal cells (**Figure 1A and 1B**).[23] In our clinical trial, the experimental ointment will contain 1 milligram of gentamicin per milliliter of ointment vehicle.

ii. ***Injection of gentamicin into the high dermis of RDEB patient skin****:* In addition, we will identify target 2.0 cm x 2.0 cm areas of unwounded intact skin within areas prone to blister formation and intradermally inject commercially available sterile gentamicin solution (40 mg/ml in saline). A similar control area will be injected with equal volumes of saline. We will inject 200 μl (8 milligrams) into each site once on day 0 and once again on day 1. A single injection will be administered to the site on each of the two days. The total dose will be 16 milligrams injected into the upper dermis where it will contact the patient's dermal fibroblasts and basal keratinocytes. In our published *in vitro* read-through study, each 1 cm^2 of cultured cells was exposed to 400 μgs/ml of gentamicin that showed read-through activity with no cytoxocity. For the proposed intradermal study, we will be using a total dose approximately 5-fold greater than the *in vitro* dose.

iii. Initial and follow-up Parameters: Prior to any treatment, the RDEB patients will be subjected to a 9 mm shave biopsy of intact skin that will be divided into 3 parts and evaluated for H&E histology, transmission electron microscopy and direct immunofluorescence for C7 expression. At 1 and 3 months after gentamicin treatment of RDEB erosions or intact skin in blister prone areas, we will biopsy the treated sites and repeat the histological, ultrastructural and C7 expression evaluations. For the assessment of C7 expression at the DEJ by immunofluorescence (IF), 5 □icron cryosections will be probed with anti-C7 polyclonal antibodies to the NC1 and NC2 domains of C7. The increased expression of the NC2 domain of C7 at the DEJ of gentamicin-treated skin or erosions will serve as one major "milestone" in this study since it would indicate PTC read-through and restoration of a full-length C7. The third part of the biopsy will be evaluated ultrastructurally, and AFs will be enumerated by computer-assisted morphometry. These studies will assess if there is restoration of normal AFs. Skin sections from normal human subjects will serve as positive controls, while skin sections from the vehicle control site will serve as negative controls.

C. Patient clinical assessment: *Skin Erosion Sites*: Patients will be blinded to the gentamicin and vehicle treatments of the Experimental Site and Control Site. Each week, the patients will assess the sites and grade their healing as follows: - 1 = enlargement of the erosion compared to its initial size; 0 = no change in the size of the erosion; +1 = partial healing and a smaller erosion than its initial size, and +2 = complete closure of the wound.

Secondly, baseline photographs of the erosions will be generated and the area of the erosions calculated by computer-assisted planimetry. Identical assessments will be made at 1 and 3 months post treatment. Therefore, a second "milestone" for this study will be decreased surface areas of gentamicin-treated erosions compared with vehicle control-treated erosions.

Evaluation of RDEB intact skin treated by intradermal injections of gentamicin: Patients and Investigators will be blinded to the treatments of the Experimental Area and Control Area. Each week, the patient will evaluate the areas and grade them as follows: - 1 = new blister or erosion formation in the site, and +1 = no new blisters or erosions. At USC visits at 1 and 3 months, biopsies will be obtained from the sites and evaluated as above for the expression of C7 at the DEJ and enumeration of AFs.

D. Evaluation of Patients' Safety: Patients will have baseline histories, review of systems (ROS), vital signs (including weight) and physical examinations on Day 0 before treatment, and at Day 1 (one day after treatment) and then at 1 and 3 months during their visits to USC. At these same time points, blood tests will be performed and include a complete blood count, electrolytes, liver enzymes, erythrocyte sedimentation rate, creatinine, and BUN. Creatinine clearances will be also calculated, and the patient's treatment sites will be evaluated for erythema, edema, blistering and erosions. At Day 0, 1 month and 3 months after treatment, audiometry evaluations will be done. Patients will complete a ROS questionnaire daily at home, and be telephoned weekly by a USC study member inquiring about any new signs, symptoms, or ROS changes.

E. Characterization of Immune Responses to Gentamicin-Induced C7: Our study patients all express lower levels of C7 including the NC1 domain, which is the most antigenic domain of C7. Therefore, with the exception of patient B, we doubt that reading through the patient's PTC will induce a protein that is viewed as antigenic by the patient's immune system. Nevertheless, we hope that gentamicin will generate a functional, rather than non-functional, species of C7. To evaluate if this change triggers an immune response and generates anti-C7 antibodies, patient serum will be obtained at baseline, 1 month, and 3 months for evaluation of anti-C7 antibodies by salt-split IIF and ELISA. If a patient develops antibodies to C7, we will then examine their skin for C7 antibody deposits by DIF.

F. Discussion on Potential Toxicities Associated with Topical or Intradermal Gentamicin.
In our *in vitro* skin cell cultures, read-through of non-sense mutations in the *COL7A1* gene occurred at concentrations of gentamicin within the 0.2 - 0.4 mg/ml for cultured human keratinocytes and cultured human fibroblasts. The commercially available FDA-approved topical gentamicin products (Gentamicin 0.1% Topical Cream) are 1 mg/ml, and they have been used for years in open skin wounds of patients with no untoward side effects such as ototoxicity or nephrotoxicity. In fact, we have used these agents ourselves in a number of RDEB patients and have had no untoward side effects. We believe that if there were a significant incidence of ototoxicity or nephrotoxicity from topical 0.1% gentamicin cream or ointment that these products would have been pulled from the market by the Federal Drug Administration (FDA) long ago. For infections in patients with normal renal

function, the recommended systemic gentamicin doses are between 3 mg - 5 mg per kilogram body weight per day with the duration of treatment being 10 days. In a 70 pound child (32 kilograms) such a dose would be between 96 mg and 160 mg per day or a total dose course between 960 mg and 1600 mg.

One milliliter of ointment covers liberally a 4-cm^2 area of skin or wound. If we have one such wound or 4 cm^2 test sites and applied one milliliter of 0.1% gentamicin ointment to these sites the dose would one milligram per application. If we apply this amount three times a day, the total daily dose to the skin would be 3 milligrams per day. If we apply the gentamicin for 14 days, the total dose will be 42 milligrams to the skin. Topical application of gentamicin to open wounds generates negligible amounts of gentamicin in the blood stream. Nevertheless, for arguments sake, lets say that 100% of the topically applied gentamicin went into the blood stream. Then, the worse case scenario would be that the subject would have a total systemic exposure of gentamicin of 42 milligrams *vis a vis* the minimal therapeutic dose of gentamicin of 960 mg. Given that in reality only a very small amount of topical gentamicin enters the blood stream, we believe our proposed trial of topical 0.1% gentamicin ointment is very safe and will have minimal risk of nephrotoxicity.

To confirm topical gentamicin safety with some objective data, a recent publication in the *New England Journal of Medicine* shows the safety of topically applied gentamicin. Please see the paper by Salah AB et al. entitled "Topical Paromycin With or Without Gentamicin for Cutaneous Leishmaniasis", *New England Journal of Medicine* volume 368, pages 524 - 532, 2013. These investigators applied gentamicin 0.5% cream (five times stronger than the commercially available topical 0.1% gentamicin cream we plan to use in our study) in combination with 15% paromomycin to ulcerative cutaneous leishmaniasis lesions once daily for 20 days. There was no evidence of any renal toxicity or ototoxicity.

For the intradermal gentamicin injection arm of the study, we will be injecting experimental areas totaling 4 cm^2. These areas will be injected intradermally with 200 microliters of commercially available gentamicin solution, 0.04 milligrams per microliter. Each injection will be 8 milligrams. If we inject once a day for a total of 2 days, the total dose to each subject will be 16 milligrams. In reality, none or a small fraction of the intradermally injected gentamicin will enter the subject's blood stream. Nevertheless, if we assumed that 100% of the intradermal injections went to the patient's blood stream, the total possible dose of gentamicin to which the patient is exposed would be 16 milligrams. Again this is markedly lower than the minimal recommended therapeutic dose of gentamicin of 960 milligrams. Therefore, like our proposed experiments with topical gentamicin ointment, we believe the proposed experiments with intradermally injected gentamicin are extremely safe.

We believe the proposed experiments with topical and intradermally injected gentamicin are very safe, particularly since only a small fraction, if any, of the gentamicin administered in these ways will enter the patient's blood stream. Nevertheless, as outlined in our experimental protocol, will carefully monitor the subjects' creatinine, BUN and creatinine clearance for any early signs of renal toxicity from the topically applied gentamicin ointment. Moreover, we will have a pediatric nephrologist Dr. Lawrence Opas, the Chief of Pediatrics at LAC + USC Medical Center and a seasoned pediatric nephrologist advise us throughout this study and review the six subjects' laboratory data.

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Timeline, Milestones, Expected Measurable Outcomes and Deliverables:
Timeline: This is an 12-month grant. We will enroll 6 RDEB patients (5 with nonsense mutations and one without a nonsense mutation) that we have characterized and published before as shown in Table I. We will re-evaluate their clinical disease, re-assess their RDEB disease classification, assess their basal levels of C7 and AFs, and ensure that they do not have any of our exclusion criteria. We will commence the study with Aim 1 to evaluate the safety and efficacy of topical and intradermal gentamicin for RDEB patients. Aim 1 will be completed in 12 months.

11/1/15 – 12/26/15: Write and submit an IRB for USC institutional approval and prepare RDEB patients for the trial.
1/1/16 – 3/31/16: Invite 6 RDEB patients for initial screening and begin Aim 1. We will also analyze the patients' baseline biopsies for C7 and AF expression.
4/1/16 – 6/30/16: Patients will return to USC for a one-month follow-up visit, evaluation, photography paired with computer-assisted planimetry, and skin biopsies to evaluate C7 and AF expression.
7/1/16 – 9/30/16: Patients will return to USC for a three-month follow-up visit, evaluation, photography paired with computer-assisted planimetry, and skin biopsies to evaluate C7 and AF expression. A data analysis of Aim 1 will be completed.
10/1/16 – 10/30/16: We will write a final report and a manuscript for publication.

Milestones: By 12 months into the grant we will have identified and completed the evaluation of 6 RDEB patients who have received topical and intradermal injections of gentamicin.

Expected Measurable Outcomes and Deliverables: Our Outcome-Safety Parameters are un-ambiguous. The patients' vital signs, physical examinations, review of systems, diaries, weekly telephone interviews, and face-to-face evaluations are all readily measurable and easy to document. Possible renal toxicity and ototoxicity from gentamicin exposure will be ascertained by means of serial creatine clearances, audiometry results, and serial signs and symptoms as reported by the patients. These parameters are deliverable with no technical hurdles.

Our Outcome Laboratory Parameters are objective and without technical hurdles. The patients either will or will not have increased C7 and AFs at the DEJ of gentamicin Treated Sites compared with vehicle Control Sites when evaluated by straight forward, objective measures of fluorescence intensity according to Wong and co-workers[5] and computer-assisted electron micrographs. These parameters are objective and without technical hurdles. We will define "a significant restoration" of both functional C7 and AFs as an increase of 35% or more above baseline. This will be the threshold for our Minimal Pharmacological Dose (MPD). We chose this criterion based on the following: 1) A morphometric analysis of AF numbers in RDEB skin revealed a decrease of at least 77% below that of normal skin. 2) Studies with RDEB-like C7 knockout mice showed that restoration of AF numbers to 35% of normal was sufficient for reasonable epidermal-dermal adherence, and 3) Family members of RDEB patients who are heterozygous carriers of a *COL7A1* null mutations and have 50% of the normal complement of C7 and AFs are phenotypically normal with no skin fragility or mechanobullous disease. Taken together, these data indicate that about 35% of the normal levels of C7 and AFs are necessary for good epidermal-dermal adherence, and this should be the goal of our gentamicin therapy.

Our Outcome Clinical Parameters are readily deliverable and without technical hurdles, but do rely on some blinded patient and blinded physician evaluator's subjective measurements. Because the patients will not be aware of which sites received gentamicin or vehicle, they will be blinded. Therefore, the patient diaries of new

lesions and comparisons between sites should be valid. Weekly telephone contact by us should reinforce the patients' compliance in documenting these parameters.