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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.



Rapamycin selectively inhibits expression of an inducible keratin (K6a) in human keratinocytes and improves symptoms in pachyonychia congenita patients

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ABSTRACT

Background: The macrolide sirolimus (rapamycin) selectively blocks translation of mRNAs containing a terminal 5' oligopyrimidine (TOP) tract by altering the activity of mammalian target of rapamycin (mTOR) and inhibiting downstream mTOR pathway components involved in TOP mRNA translation. The skin disorder pachyonychia congenita (PC) is caused by mutations in the inducible keratins (K) including K6a, K6b, K16 and K17. Published sequence data suggest the 5' untranslated regions of K6a and K6b mRNAs contain 5' TOP motifs and therefore may be sensitive to rapamycin treatment.

Objective: Determine if mTOR inhibitors (rapamycin, temsirolimus or everolimus) are viable drug candidates for treatment of PC and other disorders caused by inappropriate expression of K6a and K6b.

Methods: 5' RACE analysis was used to map the transcriptional start sites for K5, K6a, K6b, K14, K16 and K17. The sensitivity of these keratins to mTOR inhibitors was determined by Western and qPCR analysis following treatment of a human HaCaT keratinocyte cell line with rapamycin, temsirolimus or everolimus. A small off-label study was undertaken using orally administered rapamycin in three PC patients and the effects were monitored by clinical examination, photography, a validated Dermatology Life Quality Index (DLQI) and a pain and activity diary.

Results: Sequence comparison and 5' RACE analysis of the 5' untranslated regions of K6a and K6b revealed putative TOP regulatory elements. Treatment of a human HaCaT keratinocyte cell line with mTOR inhibitors (rapamycin, temsirolimus or everolimus) resulted in selective K6a repression. Furthermore, treatment of this HaCaT cell line with siRNAs targeting components of the mTOR pathway altered the levels of K6a expression. To test the ability of rapamycin to ameliorate PC symptoms, an off-label study was conducted. PC patient clinical responses to oral rapamycin showed a therapeutic response in callus character as well as subjective improvement. Of particular note, rapamycin greatly reduced the presence of painful cutaneous thromboses after reaching therapeutic serum levels. The well-known rapamycin side effects led to the early withdrawal of all of the patients from the study.

Conclusion: Rapamycin selectively blocks K6a expression in human keratinocytes. The improvement of symptoms in PC patients following rapamycin treatment suggests rapamycin (or rapamycin analogs) may be a therapeutic option, particularly if topical formulations can be developed that avoid the side effects associated with systemic administration.

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1. Introduction

Regulation of K6a and/or K6b expression has been suggested to occur post-transcriptionally, potentially at the level of mRNA translation [1]; functional K6a and K6b mRNAs are found throughout the epidermis [1] but are not translated into protein with the exception of palm and plantar skin [2]. This finding

suggests a regulatory mechanism exists to prevent translation of these mRNAs and yet allow rapid expression when needed and an appropriate signal is present [1].

Translational regulation is often mediated through 5' untranslated motifs. The 5' terminal oligopyrimidine (TOP) motif [3] has been shown to confer regulation at the translation level in a growth-dependent fashion [4]. Messenger RNAs containing these TOP motifs (4–14 pyrimidines adjacent to the 5' cap site) [5] have been shown to be post-transcriptionally regulated at the level of mRNA translation [5–7]. Furthermore, treatment with the immunosuppressant macrolide sirolimus (rapamycin) has been shown to specifically downregulate TOP mRNA translation [8–10]. Rapamycin forms a

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complex with the FK binding protein complex (FKBP-12), which binds to and inhibits the mammalian target of rapamycin (mTOR), resulting in inactivation of p70 S6 kinase (p70^{S6K}) as well as limits the availability of eukaryotic translation initiation factor 4E (eIF-4E), the mRNA 5' cap binding protein [4,11–13]. Diminished levels of eIF-4E, thought to be the rate limiting factor in translation initiation, result in reduced mRNA translation, particularly of those mRNAs that do not compete well for the limiting amounts of eIF-4E (e.g., TOP mRNAs). The activity of eIF-4E is regulated by eIF-4E binding proteins (4E-BPs), which comprise a family of repressor phosphoproteins. Phosphorylated 4E-BPs are unable to bind eIF-4E, allowing translational initiation. Upon inactivation of mTOR, 4E-BPs are not phosphorylated and remain bound to eIF-4E [4,6,14]. Although the first TOP mRNAs identified were mainly ribosomal proteins and translation initiation factors, additional non-ribosome-related members have been identified [3].

In this report, we present experimentally determined 5' UTR sequences for K5, K6a, K6b, K14, K16 and K17 and show that rapamycin and other members of the macrolide family, including temsirolimus (also known as Torisel[®] or CCI-779) and everolimus (Afinitor[®], Certican[®] or RAD001), downregulate expression of K6a, which, along with K6b, contains a 5' UTR oligo pyrimidine element. Furthermore, we present results showing that siRNA-mediated downregulation of mTOR pathway components affect K6a expression. The demonstration that rapamycin treatment inhibits K6a expression in tissue culture cells supported a small off-label use of orally administered drug (Rapamune[®], Wyeth Pharmaceuticals) to treat pachyonychia congenita (PC). PC is a rare autosomal dominant skin disorder caused by one of several dominant negative mutations in the inducible keratins K6a, K6b, K16 and K17 that disrupt intermediate filament formation [15,16] resulting in symptoms that include thickened dystrophic nails, palmar and plantar hyperkeratosis, leukokeratosis, and follicular hyperkeratosis [17]. We present the results of this study, which suggest that rapamycin improves the clinical course of PC.

2. Materials and method

2.1. Cell culture

Human HaCaT-td keratinocytes (subcloned from HaCaT keratinocytes provided by W.H. Irwin McLean and Frances J.D. Smith [18]) were maintained in DMEM (CAMBREX/BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (HyClone, Logan, UT), supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate (growth medium).

2.2. Rapamycin treatment of human keratinocytes

Rapamycin (Sigma, St. Louis, MO), temsirolimus (LC Labs, Woburn, MA) and everolimus (Sigma) were dissolved in DMSO to a final concentration of 1 mM, aliquoted and stored at –20 °C. HaCaT-td cells were seeded at 4000–8000 cells/well in a 48-well plate in 500 μ L growth medium such that cells reached 90–95% confluency when harvested. The cells were allowed to recover for 24 h before replacing the medium with growth medium containing between 0.01 and 100 nM macrolide.

2.3. Western blot analysis

HaCaT-td keratinocytes treated with rapamycin were washed twice with PBS, lysed with NuPAGE 1 \times loading dye/solubilization buffer (lithium dodecyl sulfate buffer, Invitrogen, Carlsbad, CA) supplemented with 100 mM DTT, subjected to electrophoresis in NuPAGE Novex 4–12% bis-Tris gels (Invitrogen) and electroblotted to nitrocellulose (Invitrogen). K6a expression was detected by

KA12 primary antibody (Progen Biotechnik GmbH, Heidelberg, Germany) and goat anti-mouse IgG-alkaline phosphatase secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). It should be noted that KA12, like all available K6 antibodies, cannot distinguish between K6a and K6b isoforms. K5 expression was detected by primary antibodies generously provided by Jiang Chen and Dennis Roop (University of Colorado) and guinea pig anti-rabbit IgG-alkaline phosphatase secondary antibody (Santa Cruz Biotechnology). K14 expression was detected by primary antibody (LL001) generously provided by Birgit Lane (BIOPOLIS, Singapore) and goat anti-mouse IgG-alkaline phosphatase secondary antibody. K17 expression was detected by primary antibody from Sigma (C9179-2ML) and goat anti-mouse IgG-alkaline phosphatase secondary antibody. eIF-4E and 4E-BP1 expression patterns were detected by primary antibodies obtained from Cell Signalling Technology (Danvers, MA) and guinea pig anti-rabbit IgG-alkaline phosphatase secondary antibody. All blots were visualized by the NBT/BCIP system (Promega, Madison, WI) and subsequently reacted with a primary antibody specific to lamin A/C (Upstate USA Inc, Charlottesville, VA) and goat anti-mouse IgG-alkaline phosphatase secondary antibody to show equal lane loading and absence of generalized inhibition resulting from rapamycin or siRNA treatment. Band intensities were quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>). The reported values were generated by dividing normalized K6a band intensities by the normalized K5 intensities.

2.4. Treatment of HaCaT-td keratinocytes with siRNAs

HaCaT-td keratinocytes were transfected with RNAiMAX (Invitrogen) according to the manufacturer's instructions for "reverse transfection." Twelve pmoles of SMARTpool[®] siRNAs targeting eIF-4E and 4E-BP1 and control siRNAs targeting K6a (reported as WT.12 from Ref. [19]) and eGFP were diluted with 50 μ L optiMEM medium in a well of a 48-well plate (all siRNAs were provided by Thermo Fisher Scientific, Dharmacon Products, Lafayette, CO). One microliter of RNAiMAX lipofectamine was diluted in 50 μ L of optiMEM medium and immediately added to the nucleic acid solution and incubated at 21 °C for 20 min. Trypsinized HaCaT-td cells (2×10^4 cells in 500 μ L growth medium) were then added to the well and gently mixed prior to incubation. Samples were analyzed by Western blot as described above.

2.5. Analysis of mRNA transcriptional start sites

The 5'-RACE-ready cDNA was generated by following the protocol described in the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA). Specific PCR amplification of K5, K6a, K6b, K14, K16 and K17 from total RNA isolated from skin (Stratagene, La Jolla, CA) and/or HaCaT-td keratinocytes was accomplished using the universal upstream primer and the following gene-specific downstream primers: K5 (5' CTC ATC CAT CAG TGC ATC AA), K6a (5' GGG TGC TCA GAT GGT ATA GAG), K6b (5' TTT CTT CTC AGA ATT ATG GCA), K14 (GAA GTA GGG ACT GTA GTC T), K16 (5' AGA CCT CGC GGG AAG AAT A), and K17 (TCC ACA CTC AGG CGC AGG GCC TG). DMSO (10% final concentration) was added to the total RNA samples (prior to heat denaturation) in order to remove secondary structure and facilitate reverse transcription of full-length K5 and K17 mRNA. The PCR products were ligated into the pCR[®]II TOPO-TA vector (Invitrogen) and sequenced using the SP6 promoter primer.

2.6. RTqPCR analysis

RNA was isolated from treated HaCaT-td cells 48 h after treatment with rapamycin using the RNeasy Mini Kit (Qiagen,

Valencia, CA) and reverse transcribed using random hexamer priming with the First Strand Synthesis Kit (Invitrogen). Real time PCR was performed using the TaqMan Universal PCR Master Mix and the ABI standard 7500 procedure from Applied Biosystems (Foster City, CA). Lamin A/C (HS00153462_m1), GAPDH (Hs99999905_m1), K5 (Hs00361185_m1), K6b (Hs00749101_s1), K14 (Hs00559328_m1) and K17 (Hs00373910_g1) inventoried TaqMan gene expression assays were obtained from Applied Biosystems. The K6a assay was custom designed and will be described elsewhere (Hickerson et al., manuscript in preparation). The data were analyzed with the Applied Biosystems Sequence Detection software (version 1.4) and reported as relative quantitation (RQ). All data points reported are the mean of 3 replicate assays and error is reported as standard error.

2.7. Off-label clinical study

Prior to initiation of therapy, pregnancy tests were performed in female patients (oral contraceptive prophylaxis was in use in all cases) and all three patients received a pre-therapy evaluation including: complete blood count (cbc), complete metabolic panel (CMP; sodium, potassium, chloride, carbon dioxide, anion gap, blood urea nitrogen, creatinine, glucose, alanine aminotransferase, aspartate aminotransferase, calcium, total protein, albumin, bilirubin), lipid panel (cholesterol, triglycerides, high density lipoprotein, low density lipoprotein, very low density lipoprotein), urinalysis (UA) with microanalysis, posteroanterior and lateral chest X-ray. The starting dose was 2 mg Rapamune[®] (Wyeth Pharmaceuticals Inc.) per day and trough levels were obtained at

steady state after 2 weeks. The dosage was increased every 2 weeks until trough levels reached a therapeutic range of 9–12 ng/mL. Patients were re-evaluated for effects and side effects every 2 weeks and photographs of plantar calluses were taken to document their progress. Laboratories were performed which included: rapamycin trough level, UA with microanalysis, cbc with differential, CMP, lipid panel, and a pregnancy test when indicated. Patients also completed a daily pain diary rating their level of pain on a scale of 1–10 several times per day and a biweekly validated life quality evaluation (DLQI) at each clinic visit.

3. Results

Based on previously published primer extension data reporting the K6a transcriptional start site to occur at a cytosine residue 48 nucleotides upstream of the translation start site within an extended motif consisting of 21 pyrimidines [2], HaCaT-td cells were treated with rapamycin in order to determine if expression of keratins that contain putative TOP regulatory elements can be selectively downregulated. HaCaT-td keratinocytes do not express detectable levels of K6b (F.J.D. Smith personal communication and our unpublished results); therefore, any K6 expression observed by Western blot is predominantly due to K6a. As no antibodies that can distinguish the K6a and K6b isoforms are available, this cell line lacking K6b expression allows for effects on K6a to be assayed. Fig. 1A shows that K6a expression is inhibited in a dose-dependent fashion at low rapamycin concentrations ($IC_{50} \sim 0.5$ nM). A time-course analysis revealed that inhibition begins 24 h post-treatment (Fig. 1B). Little or no effect on K6a mRNA levels was observed

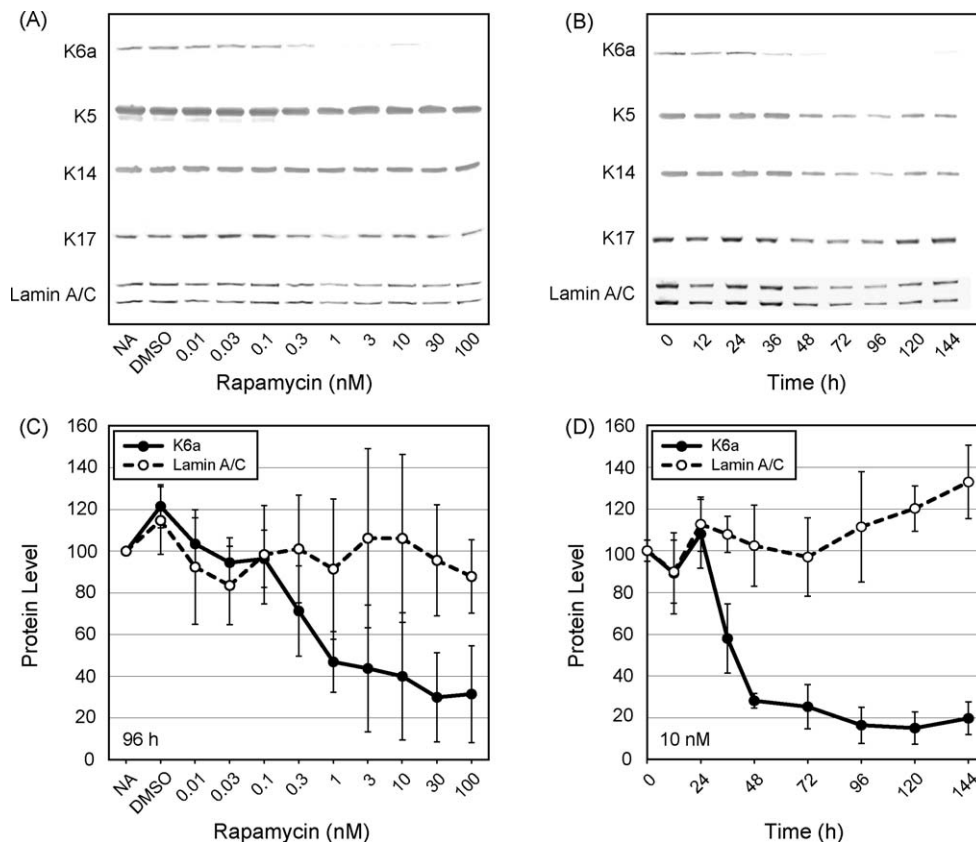


Fig. 1. Rapamycin treatment of human keratinocytes results in downregulation of K6a expression. (A) Rapamycin dose response. HaCaT-td cells were treated with increasing concentrations of rapamycin as indicated and harvested at 96 h. (B) Timecourse. HaCaT-td cells were treated with 10 nM rapamycin and harvested at the indicated time points. "NA" and "DMSO" correspond to no treatment and vehicle control, respectively. At the indicated times, cells were harvested and lysed in SDS-PAGE loading buffer, subjected to denaturing SDS-PAGE analysis and electroblotted to nitrocellulose as described in Section 2. K5, K6a, K14, K17 and lamin A/C expression was detected by specific antibodies and visualized by the NBT/BCIP system. The data were quantitated using ImageJ software (panels C and D) and normalized to 100. The plotted values for K6a and lamin A/C were obtained by dividing these normalized values by normalized K5 values. Error bars represent standard deviation.

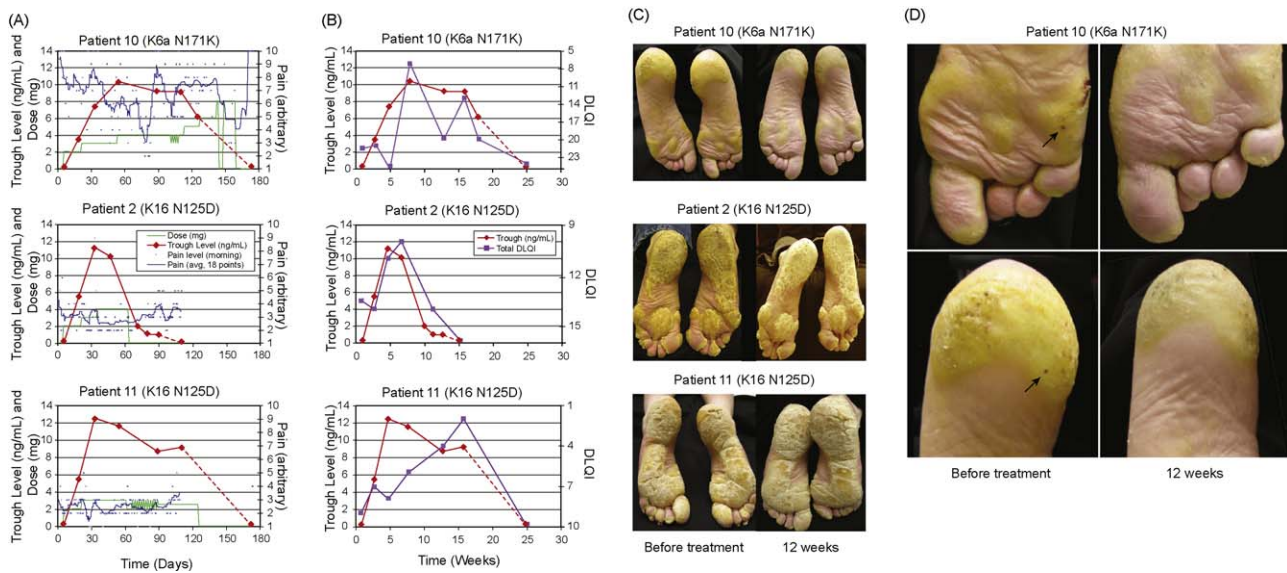


Fig. 4. Treatment with rapamycin results in clinical improvement in three pachyonychia congenita patients. (A) Three genotyped PC patients (K6a or K16 mutations as indicated) were treated with Rapamune[®] (Wyeth Pharmaceuticals) at the indicated doses. Rapamycin trough levels (red line) were measured biweekly with a target therapeutic range of 9–12 ng/mL. As the half-life of rapamycin is 79 ± 12 h in healthy volunteers [39], trough levels were assumed to be zero (dotted line) 2 weeks after discontinuation of the drug (>4 half-lives). Arbitrary pain levels were based on a 1–10 scale (10 most painful) and reported as either the morning level (upon waking—therefore not as heavily influenced by daily activity; blue dots) or the running average of morning pain levels (18 data points averaged; blue line). The daily dose is plotted in green. (B) The Dermatology Life Quality Index (DLQI) questionnaire was utilized to assess the impact of rapamycin treatment on daily activities. The Y-axis DLQI data (purple line) is inverted to allow easier comparison of increased quality of life with blood trough level (red line). (C) Before-and-after rapamycin treatment photographs of plantar hyperkeratosis, blisters and cutaneous thromboses. (D) Enlarged view of the photos of Patient 10 from panel C (arrows indicate capillary thromboses).

Although the *in vitro* data predict that K6a, and potentially K6b, are the only inducible keratins that are TOP mRNAs, and thus directly sensitive to rapamycin treatment, all adult PC patients (including non-K6 patients) in proximity to the University of Utah (study location) participated in the study. It should be noted that reduction in the levels of K6a and K6b proteins has been shown to be accompanied by a selective decrease in K16 protein expression in K6a/K6b null mice [23], suggesting that reduction of one of the proteins normally found in a keratin pair (e.g., K6a), may lead to reduction in partner (e.g., K16) protein levels. The lack of severe phenotypic effects in K6a/K6b null mice suggests that reduction of K6a and/or K6b in patients following rapamycin treatment may be compensated by functional keratin redundancy [23–25].

Rapamune was given in the standard fashion following typical recommendations for use as an adjunct therapy in renal transplantation [26] as described in Section 2. Participants (#10, #2 and #11) received Rapamune for 159, 110, and 125 days, respectively, and reached maximal trough levels (i.e., blood concentrations) of 10.4, 11.3 and 12.5 ng/mL, respectively, with maximal doses of 8, 4 and 3 mg/day, respectively.

In addition to clinical examination, the patients completed a daily pain diary, rating their level of pain on a scale of 1–10 twice daily and a validated life quality evaluation (DLQI) [27,28] at the time of their biweekly clinic visit (summarized in Fig. 4). Patient-reported pain (morning assessment) and DLQI scores are plotted over time with respect to trough level (Fig. 4A and B). All patients experienced side effects that ultimately resulted in discontinuation of the study. Patient 10 developed diarrhea and aphthous ulcers, Patient 2 developed gastrointestinal distress with loss of appetite and Patient 11 developed an acneiform follicular eruption. None of the patients experienced any laboratory abnormalities or serious side effects. Significantly, in spite of these side effects arising from systemic administration of Rapamune, all three patients reported increased life quality (as reflected by decreasing DLQI scores; note inverted axis in Fig. 4B) that parallels the rapamycin trough level.

In addition to subjective improvement of plantar pain (Fig. 4A) and improved quality of life (Fig. 4B), Patient 10 (harbors the K6a N171K mutation) also demonstrated important clinical changes in the plantar calluses. Fig. 4C shows photographs taken of the plantar keratoderma before initiation of systemic Rapamune therapy and 12 weeks into the treatment course. The photographs demonstrate decreased keratoderma following treatment. However, this finding may not be a direct effect of Rapamune treatment. This patient has long noted that the level to which it is possible to remove callus is determined by the level at which the blade reaches the superficial capillaries and presumably the associated pain fibers. At the 12-week time point, these cutaneous thromboses had regressed relative to baseline (see Fig. 4D); therefore, the patient was able to remove more callus down to the level of the regressed structures.

No changes were observed in the characteristic of PC nails during the study. It should be noted that only minor keratin-related changes, including fragile nails have been noted in some patients undergoing rapamycin treatment to prevent rejection of organ transplants [29]. The length of treatment (2–5 months) in this study may have been insufficient to observe changes in nail composition.

4. Discussion

As basal-layer keratinocytes differentiate and move through the epidermis to the *stratum corneum* they become anucleate and lose the ability to regulate gene expression at the transcriptional level. Previous research suggests that K6a and K6b gene regulation may occur at the level of mRNA translation during the differentiation process [1,2]. The 5' UTRs of the inducible keratin mRNAs (K6a, K6b, K16 and K17) contain multiple and extensive polypyrimidine tracts, including at or near the predicted 5' transcription start sites (based on genomic and mRNA sequences; see Fig. 2, boxed in red). However, our experimentally determined start sites were upstream of the previously determined or predicted sites, yet still embedded in a polypyrimidine tract (TOP regulatory elements)

and therefore are expected to be sensitive to rapamycin treatment. The experimentally determined start sites for K16 and K17 were also found upstream of the predicted start sites and 9 or 13 nucleotides upstream of polypyrimidine tracts, respectively, and are therefore not expected to act as TOP mRNAs. Indeed, Western blot analysis confirmed rapamycin sensitivity of K6a in HaCaT-td cells, while K17 was unaffected (see Fig. 1). K6b and K16 were not detected; however, based on the 5' UTR sequence data, K6b, but not K16, is expected to be rapamycin sensitive.

An off-label study of oral Rapamune for treatment of a small number ($n = 3$) of pachyonychia congenita patients was conducted. There are currently no approved, effective treatments for this disorder. However, a siRNA-based treatment for PC, which targets the N171K mutation on the dominant K6a mutant allele without affecting the wildtype allele [19], is in clinical trials and shows promise if improved "patient-friendly" delivery technologies can be found [30]. Rapamune was administered orally, and a daily pain diary and DLQI questionnaire as well as biweekly clinical evaluations were utilized to assess effects of treatment. The DLQI was used to determine the impact of Rapamune treatment on daily activities ("life quality"). The results summarized in Fig. 4B show a correlation between rapamycin trough levels and DLQI scores (R^2 values were 0.40, 0.75 and 0.34 for Patients 10, 2 and 11, respectively). As might be expected, the correlation between rapamycin trough levels and subjective pain scale was generally more variable than that of the DLQI scores (R^2 values were 0.67, 0.10 and 0.13 for Patients 10, 2 and 11, respectively, Fig. 4A).

Overall, the clinical data suggest that oral Rapamune was able to reduce the pain in all three PC patients. Of particular note for Patient 10 is the temporary discontinuation of Rapamune (days 145–148 due to concerns regarding diarrhea while traveling) during which time a trough level was not obtained. This period was associated with a high level of recurrent pain followed by decreased pain when drug treatment was re-initiated and assumed to reach therapeutic levels (trough level was not obtained). The simple pain scale used in this study was not as reliable as the DLQI in adequately capturing the overall subjective experience of pain, particularly for Patients 2 and 11. Both of these patients reported that they felt their pain was improved, but the pain scores did not reflect their general impression. These patients reported that this was due in part to the increased activity that spontaneously occurs when pain decreases (i.e., unless a complete "cure" is achieved, pain levels may never decrease as patients may increase activity levels until a similar pain threshold is reached). An improved pain scale is currently under development, which incorporates activity levels into the subjective reporting system.

Patient 10 (harboring the K6a N171K mutation) may have responded better to treatment with rapamycin as K6a contains a putative TOP regulatory element. The other patients enrolled (harboring the K16 N125D mutation) also responded but to a lesser degree. This is consistent with a model in which rapamycin leads to direct downregulation of K6a (a TOP mRNA) but not K16 (no TOP regulatory element). The clinical findings in patients harboring K16 mutations may result from a possible feedback regulatory mechanism in which K16 levels are reduced following downregulation of K6a [23].

Although rapamycin has been reported to decrease chronic pain by reducing the sensitivity of myelinated nociceptors known to be important for the increased mechanical sensitivity that follows injury [31], this mechanism does not account for the other clinical findings, namely the reduction or resolution of cutaneous thromboses in Patient 10, which was perhaps the most striking finding in this study (see Fig. 4D, plantar thromboses were not found in Patients 2 and 11 before or during this study). This regression may be due to rapamycin's anti-angiogenic properties including reduced production of VEGF and blockage of VEGF-

induced endothelial cell signaling [32]. This anti-angiogenic property of rapamycin and rapamycin analogs has been exploited for treatment of cancers including renal [33,34].

The lack of long-term tolerance to the orally administered form of rapamycin (due to its well-known side effects) and the difficulty in drug dosing (i.e., variable absorption requiring frequent trough level measurement) raises the question of whether a topical form, similar to that recently reported for psoriasis [35], might be advantageous. In the psoriasis study, it was shown that rapamycin in a capric acid/isopropyl myristate/benzyl alcohol topical formulation was able to penetrate human skin, and improvement based on immunohistochemical markers and reduction of psoriatic plaques was observed [35]. Although this topical rapamycin formulation showed improvement in psoriasis patients, the authors suggest that drug penetration efficiency needs to be improved in order to treat all types of skin (i.e., thicker skin and skin in which the barrier function is not compromised).

In addition to rapamycin, the effect of temsirolimus and everolimus was evaluated in HaCaT-td keratinocytes and shown to similarly downregulate K6a. Temsirolimus (Torisel[®]) is approved for treatment of renal cell carcinoma [36]. Everolimus (Certican[®]) is approved as an immunosuppressant to prevent rejection of organ transplants [37] in Europe and Australia and was recently approved (as Afinitor[®]) for treatment of renal cell carcinoma in the United States [37,38]. These rapamycin derivatives offer other treatment possibilities that may reduce the side effects associated with oral rapamycin treatment.

In summary, a novel regulatory mechanism for K6a gene expression has been identified as well as a class of drug that can target that specific mechanism. This has permitted the successful testing of a rational approach in PC patients, the results of which suggest that mTOR inhibitors may be useful agents for treating PC and other related disorders, particularly if topical formulations can be developed that reduce the side effects associated with oral administration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2009.07.008.

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