To analyze usage and availability of sun protection devices, sun protection scores (SPUS and SPAS) were created. Points were given for subjects' responses regarding usage of sunscreen, sunglasses, hats/headgear, or shade structures. Sporadic use received 0 points, occasional use received 1 point, and routine use received 2 points.

For statistical analysis, independent sample *t*-tests assuming unequal variance and χ^2 tests were used for group comparisons when appropriate. Pearson's correlation coefficient (*r*) was used for correlation analyses. A multivariate linear/logistic regression model was designed and performed for sun protection use and for sunburns. Two-tailed *P*-values < 0.05 were considered to be statistically significant. Statistical analyses were carried out using Stata Statistical Software, version 12 (Statacorp, College Station, TX).

Jennifer G. Powers[1,2], Neelam A. Patel[1], Edward M. Powers[1], Jonathan E. Mayer[3], George P. Stricklin[1,2] and Alan C. Geller[4]

[1]Division of Dermatology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA; [2]Tennessee Valley Healthcare System, Nashville, Tennessee, USA; [3]Columbia University College of Physicians and Surgeons, New York, New York, USA and [4]Harvard School of Public Health, Boston, Massachusetts, USA
E-mail: jennifer.powers@vanderbilt.edu

REFERENCES

Armed Forces Health Surveillance C (2014) Sunburn among active component service members, U.S. Armed Forces, 2002-2013. *MSMR* 21:2–6

Brown J, Kopf AW, Rigel DS et al. (1984) Malignant melanoma in World War II veterans. *Int J Dermatol* 23:661–3

Dubas LE, Adams BB (2012) Sunscreen use and availability among female collegiate athletes. *J Am Acad Dermatol* 67:876 e1–6

Garland FC, White MR, Garland CF et al. (1990) Occupational sunlight exposure and melanoma in the U.S. Navy. *Arch Environ Health* 45:261–7

Harris PA, Taylor R, Thielke R et al. (2009) Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 42:377–81

Lea CS, Efird JT, Toland AE et al. (2014) Melanoma incidence rates in active duty military personnel compared with a population-based registry in the United States, 2000-2007. *Mil Med* 179:247–53

Linos E, Swetter SM, Cockburn MG et al. (2009) Increasing burden of melanoma in the United States. *J Invest Dermatol Symp Proc* 129:1666–74

Page WF, Whiteman D, Murphy M (2000) A comparison of melanoma mortality among WWII veterans of the Pacific and European theaters. *Ann Epidemiol* 10:192–5

Ramani ML, Bennett RG (1993) High prevalence of skin cancer in World War II servicemen stationed in the Pacific theater. *J Am Acad Dermatol* 28:733–7

Rogers HW, Weinstock MA, Harris AR et al. (2010) Incidence estimate of nonmelanoma skin cancer in the United States, 2006. *Arch Dermatol* 146:283–7

Strand LA, Martinsen JI, Koefoed VF et al. (2011) Cause-specific mortality and cancer incidence among 28,300 Royal Norwegian Navy servicemen followed for more than 50 years. *Scand J Work Environ Health* 37:307–15

Woolley SD, Hughes C (2013) A young military pilot presents with a periocular basal cell carcinoma: a case report. *Travel Med Infect Dis* 11:435–7

Yamane GK (2006) Cancer incidence in the U.S. Air Force: 1989-2002. *Aviat Space Environ Med* 77:789–94

Zhou J, Enewold L, Zahm SH et al. (2011) Melanoma incidence rates among whites in the U.S. Military. *Cancer Epidemiol Biomarkers Prev* 20:318–23

*JID*Open

Dual mTOR Inhibition Is Required to Prevent TGF-β-Mediated Fibrosis: Implications for Scleroderma

TO THE EDITOR
Transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) are central mediators of fibrosis, and their overexpression contributes to the pathophysiology of scleroderma, chiefly by inducing the overproduction of extracellular matrix proteins (ECM) by dermal fibroblasts (Gay et al., 1989; Sargent et al., 2010; Bhattacharyya et al., 2012). TGF-β also promotes the differentiation of dermal fibroblasts into myofibroblasts, which are key mediators of scleroderma (Abraham et al., 2007). Thus, targeting this pathway is a reasonable strategy to treat a variety of fibrotic diseases including scleroderma, for which current treatment options are limited. Herein we explore the potential of novel mTOR inhibition as a means to block the pro-fibrotic effects of TGF-β. Recent studies have suggested a functional role of mTOR in fibrotic diseases
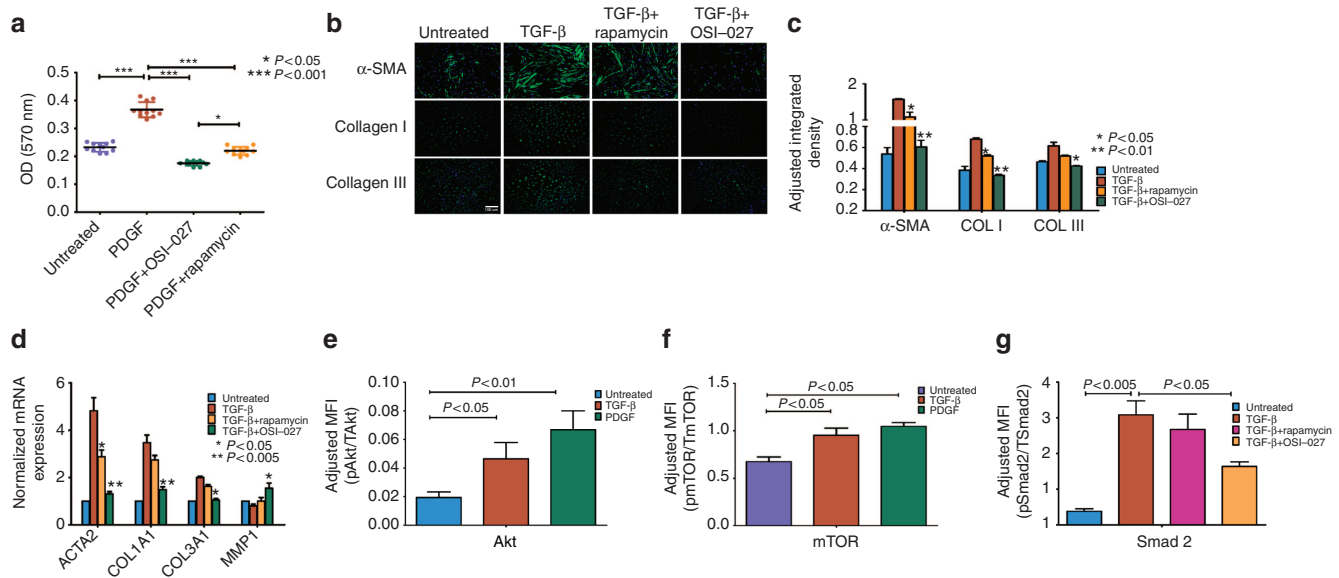
Figure 1. Comparative antifibrotic potential of OSI-027 and rapamycin. NHDFs were pre-treated with OSI-027 (5 uM) or rapamycin (10 nM) for 2 hours followed by an addition of PDGF (40 ng ml^{-1}) or TGF-β (5 ng ml^{-1}) and incubated for 3 days. Proliferation was determined by the MTT assay ($n=11$). Immunofluorescence microscopy (IF, $n=6$) and qPCR ($n=6$) were conducted to determine the expression of α-SMA, collagen I, and collagen III at protein and mRNA level, respectively. (**a**) Scatter plot showing the antimitotic effect of OSI-027 and rapamycin on PDGF-stimulated NHDFs. OSI-027 showed significantly more antimitotic effect compared with rapamycin. (**b**) Representative image showing the effect of OSI-027 and rapamycin on expression of α-SMA, collagen I, and collagen III. (**c**) Bar diagram comparing the inhibitory effect of OSI-027 and rapamycin on TGF-β-induced expression of α-SMA, collagen I, and collagen III. (**d**) Bar diagram showing the effect of OSI-027 and rapamycin on α-SMA (ACTA2), collagen I, and collagen III genes. OSI-027 showed more inhibitory effect on these genes compared with rapamycin. (**e–g**) Phosphorylation of mTOR kinases with PDGF and TGF-β. Luminex-based cell signaling assay was performed with treated and untreated cell lysates to determine molecular mechanism of mTOR inhibitors as antifibrotic agent ($n=5$). Data are represented as adjusted median fluorescence intensity (MFI) = MFI of phospho protein/MFI of total protein. Bar diagram showing significant upregulation of pAkt^{Ser473} (represents mTORC2 activity), pmTOR^{Ser2448} with PDGF, and TGF-β and a significant inhibition of TGF-β-induced pSmad2 with OSI-027. Data are represented as Mean ± SEM. All experiments were performed in triplicate. The Mann–Whitney U-test was performed to determine statistical significance. Scale bar = 100 μm. MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; NHDF, normal human dermal fibroblast; PDGF, platelet derived growth factor; qPCR, quantitative PCR.

and autoimmunity (Ong et al., 2007; Fried et al., 2008; Su et al., 2009; Raychaudhuri and Raychaudhuri, 2014). This pathway is initiated by cytokines and growth factors that induce phosphorylation of Akt$^{(Thr308)}$ and its downstream mediators mTORC1, p70S6K1, and 4E-BP1. In addition to mTORC1, mTOR is now known to comprise a second multiprotein complex, mTORC2, which positively regulates the activity of mTOR through phosphorylation of Akt^{Ser473} (Bhagwat et al., 2011). Blockade of mTORC1 has already been attempted as a treatment for scleroderma without much understanding about its molecular mechanism (Fried et al., 2008; Su et al., 2009; Yoshizaki et al., 2010). Such strategies focused only on mTORC1 and did not account for the contributions of mTORC2 (Bhagwat et al., 2011). To increase the effectiveness of mTOR blockade, dual

inhibitors targeting both mTORC1 and mTORC2 have now been developed (Bhagwat et al., 2011). Herein, we demonstrate that dual mTOR blockade can more effectively inhibit the pro-fibrotic effects of TGF-β and PDGF. These have therapeutic implications for scleroderma and other TGF-β dominant fibrotic diseases.

Antiproliferative effect of OSI-027, a dual mTOR inhibitor
The antimitotic effects of mTOR inhibition were evaluated using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. Normal human dermal fibroblasts (NHDFs) were incubated with PDGF, a known NHDF mitogen, and cultures were treated with either OSI-027 or the mTORC1 inhibitor rapamycin. OSI-027 was found to be more effective at inhibiting PDGF-induced NHDF proliferation

compared with rapamycin ($P<0.05$) (Figure 1a).

Expression of α-SMA, collagen I, and III
Apart from proliferating more, in response to TGF-β, NHDFs are known to differentiate into myofibroblasts and increase their production of ECM proteins. Thus, the expression of α-SMA (a marker of myofibroblasts) and the ECM-collagen I and III was determined by immunofluorescence microscopy (IF) following incubation with TGF-β (Abraham et al., 2007). In comparison with rapamycin, OSI-027 was more effective at inhibiting TGF-β-induced α-SMA ($P<0.01$), collagen I ($P<0.01$), and collagen III ($P<0.05$) expression (Figure 1b and c). Similarly, by quantitative PCR (qPCR), OSI-027 was found to be more effective at inhibiting TGF-β-induced upregulation of ACTA2 ($P<0.005$), COL1A1 ($P<0.005$), and COL3A1
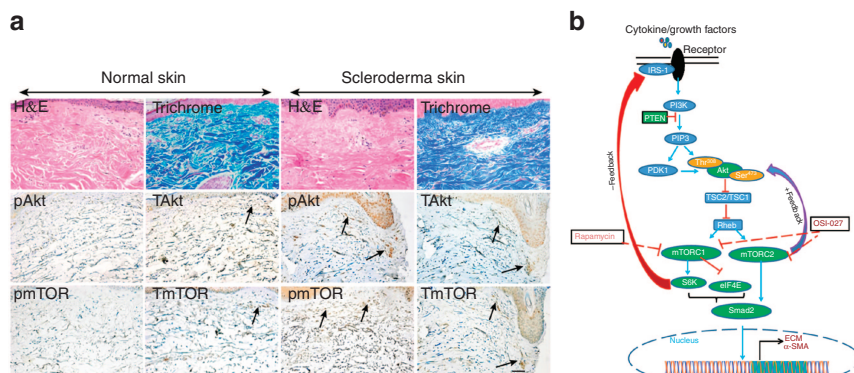
Figure 2. Differential expression of mTOR kinases in scleroderma and normal skin. (**a**) Representative image showing the differential expression of mTORC1 (pmTOR) and mTORC2 (pAkt^{Ser473}) in scleroderma and normal skin. Serial sections of scleroderma and normal skin tissues were stained with H&E, Masson's trichrome, pAkt^{Ser473}, pmTOR^{Ser2448}, Total Akt, and Total mTOR. Tissues were counterstained with toludine blue. Black arrow indicates the positively stained cells. (**b**) Schematic diagram depicting the underlying molecular mechanism of mTOR inhibitors as antifibrotic agents. Scale bar = 100 μm.

($P < 0.05$) gene expression. OSI-027 was also able to reverse TGF-β-induced downregulation of MMP1 ($P < 0.05$) (Figure 1d).

Phosphorylation of mTOR kinases and Smad2

The ability of PDGF and TGF-β to alter mTOR signaling was confirmed using a luminex-based cell-signaling assay. Figure 1e and f demonstrated that TGF-β and PDGF were able to induce significant phosphorylation of Akt^{Ser473} (representing mTORC2 activity) and mTOR^{Ser2448}. As Smad signaling is critical for TGF-β-mediated induction of ECM (Bhattacharyya et al., 2012), we determined the effect of OSI-027 on phosphorylation of Smad2. We found that TGF-β-induced phosphorylation of Smad2 was effectively inhibited by OSI-027 ($P < 0.05$) (Figure 1g). This provides an underlying molecular mechanism for the antifibrotic effects of dual mTOR inhibition.

Expression of mTOR kinases in scleroderma

On the basis of the *in vitro* data, we sought to evaluate whether mTOR was expressed in lesional skin of scleroderma patients. Immunohistochemical staining (Figure 2a) demonstrated elevated expression of pAkt^{Ser473}, pmTOR^{Ser2448}, and their respective total proteins in scleroderma compared with normal skin. Quantification of pAkt^{Ser473} was used as

a surrogate for mTORC2 activity (Bhagwat et al., 2011).

Although there are few studies suggesting the antifibrotic properties of rapamycin, chiefly a mTORC1 inhibitor (Ong et al., 2007; Fried et al., 2008; Su et al., 2009; Yoshizaki et al., 2010; Tamaki et al., 2014), to date, there have been no detailed mechanistic studies on mTOR's role in TGF-β-mediated fibrotic pathways. Moreover, in cancer biology, it is becoming increasingly evident that targeting only mTORC1 does not completely inhibit this cascade (Figure 2b). To overcome this deficiency, dual mTOR (mTORC1/mTORC2) inhibitors have been developed—e.g. OSI-027 (Bhagwat et al., 2011)—and are currently being evaluated in a phase I trial for solid cancers (https://clinicaltrial.gov/ct2/show/NCT00698243?term=OSI-027&rank=1). Herein using an *in vitro* system, we demonstrated the ability of dual mTOR inhibition to reverse TGF-β and PDGF-mediated fibrotic processes more efficiently compared with rapamycin and elucidated the underling molecular mechanism. Considering the crucial role of PDGF and TGF-β in the pathogenesis of scleroderma, our results support the development of dual mTOR inhibitors for scleroderma and other TGF-β-mediated fibrotic diseases.

Normal human dermal fibroblast (NHDF)

Third to sixth passage NHDFs (ATCC PCS-201-012) were cultured at 37 °C per 5% CO_2 in DMEM containing 10%

FBS and 1% antibiotic-antimycotic. Cells were incubated in the presence or absence of PDGF (40 ng ml^{-1}), TGF-β (5 ng ml^{-1}) with or without rapamycin (10 nM), and OSI-027 (5 uM) for 3 days, which corresponded to their optimal concentrations. To measure proliferation, the MTT reagent was added and incubated at room temperature for 2 hours. Plates were read at 570 nm (Datta-Mitra et al., 2013).

Immunofluorescence microscopy (IF)

NHDFs were processed for IF as described (Luna et al., 2011). The data are represented as adjusted integrated density using Image J (NIH, Bethesda, MD).

Luminex assay

Expression of pAkt^{Ser473}, pmTOR^{Ser2448}, pSmad2$^{Ser465/467}$, corresponding total proteins, and β-actin was determined using the Bio-Plex Pro cell signaling kit (Bio-Rad) (Lang and Sandoval, 2014). The phospho-proteins were normalized to respective total-proteins and represented as adjusted median fluorescence intensity (MFI).

Quantitative PCR

RNA was extracted using the RNeasy plus mini kit (Qiagen) and integrity determined by Agilent 2200 TapeStation. Total RNA was reverse transcribed using iScript, and qPCR was performed using customized PrimePCR plates (Bio-Rad, Hercules, CA). Reference genes used for normalization were GAPDH and HPRT1. Data were analyzed using the Bio-Rad CFX manager software (Bio-Rad) and expressed as fold change.

Skin biopsy

After obtaining IRB approved written informed consent, punch biopsies were obtained from three scleroderma patients with diffuse disease, and normal skin samples were obtained from discarded surgical skin.

Immunohistochemistry

Serial sections were deparaffinized and rehydrated and then immersed in 10 mM sodium citrate buffer. After antigen retrieval, endogenous peroxidase activity was blocked, and 10% BSA was used to

inhibit nonspecific binding. Sections were stained with antibodies with the following specificities—pAkt^{Ser473}, total Akt, pmTOR^{Ser2448}, and total mTOR. Stained tissues were incubated with a secondary antibody followed by ABC reagents and DAB (Vector Lab, Burlingame, CA). Toludine blue was used to counterstain.

CONFLICT OF INTEREST
The authors state no conflict of interest.

Anupam Mitra[1], Jesus I. Luna[1], Alina I. Marusina[1], Alexander Merleev[1], Smriti Kundu-Raychaudhuri[2], David Fiorentino[3], Siba P. Raychaudhuri[2] and Emanual Maverakis[1]

[1]Department of Dermatology, School of Medicine, University of California Davis, Sacramento, California, USA; [2]School of Medicine, University of California Davis, VA Medical Center Sacramento, Mather, California, USA and [3]Department of Dermatology, Stanford University, Redwood City, California, USA
E-mail: emaverakis@yahoo.com

REFERENCES

Abraham DJ, Eckes B, Rajkumar V et al. (2007) New developments in fibroblast and myofibroblast biology: implications for fibrosis and scleroderma. *Curr Rheumatol Rep.* 9: 136–43

Bhagwat SV, Gokhale PC, Crew AP et al. (2011) Preclinical characterization of OSI-027, a potent and selective inhibitor of mTORC1 and mTORC2: distinct from rapamycin. *Mol Cancer Ther* 10:1394–406

Bhattacharyya S, Wei J, Varga J (2012) Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. *Nat Rev Rheumatol* 8:42–54

Datta-Mitra A, Mitra A, Ray R et al. (2013) 1,25-Dihydroxyvitamin D3-3-bromoacetate, a novel vitamin D analog induces immunosuppression through PI3K/Akt/mTOR signaling cascade. *Int Immunopharmacol* 17: 744–51

Fried L, Kirsner RS, Bhandarkar S et al. (2008) Efficacy of rapamycin in scleroderma: a case study. *Lymphat Res Biol* 6:217–9

Gay S, Jones RE Jr, Huang GQ et al. (1989) Immunohistologic demonstration of platelet-derived growth factor (PDGF) and sis-oncogene expression in scleroderma. *J Investig Dermatol Symp Proc* 92:301–3

Lang WH, Sandoval JA (2014) Detection of PI3K inhibition in human neuroblastoma using multiplex luminex bead immunoassay: a targeted approach for pathway analysis. *J Biomol Screen* 19:1235–45

Luna JI, Ciriza J, Garcia-Ojeda ME et al. (2011) Multiscale biomimetic topography for the alignment of neonatal and embryonic stem cell-derived heart cells. *Tissue Eng Part C Methods* 17:579–88

Ong CT, Khoo YT, Mukhopadhyay A et al. (2007) mTOR as a potential therapeutic target for treatment of keloids and excessive scars. *Exp Dermatol* 16:394–404

Raychaudhuri SK, Raychaudhuri SP (2014) mTOR Signaling Cascade in Psoriatic Disease: Double Kinase mTOR Inhibitor a Novel Therapeutic Target. *Indian J Dermatol* 59:67–70

Sargent JL, Milano A, Bhattacharyya S et al. (2010) A TGFbeta-responsive gene signature is associated with a subset of diffuse scleroderma with increased disease severity. *J Investig Dermatol Symp Proc* 130:694–705

Su TI, Khanna D, Furst DE et al. (2009) Rapamycin versus methotrexate in early diffuse systemic sclerosis: results from a randomized, single-blind pilot study. *Arthritis Rheum* 60:3821–30

Tamaki Z, Asano Y, Kubo M et al. (2014) Effects of the immunosuppressant rapamycin on the expression of human alpha2(I) collagen and matrix metalloproteinase 1 genes in scleroderma dermal fibroblasts. *J Dermatol Sci* 74: 251–9

Yoshizaki A, Yanaba K, Yoshizaki A et al. (2010) Treatment with rapamycin prevents fibrosis in tight-skin and bleomycin-induced mouse models of systemic sclerosis. *Arthritis Rheum* 62:2476–87

A Deep-Intronic *FERMT1* Mutation Causes Kindler Syndrome: An Explanation for Genetically Unsolved Cases

TO THE EDITOR

Kindler syndrome (KS) is a distinct type of epidermolysis bullosa (EB) defined by variable levels of skin cleavage and a progressive phenotype comprising skin blistering, photosensitivity, poikiloderma, mucocutaneous scarring, and malignancies (Has et al., 2011). KS is caused by mutations in *FERMT1*, the gene encoding kindlin-1 (Jobard et al., 2003). The particular features of KS may rely on the functions of kindlin-1, which is a member of the protein family of kindlins, essential integrin activators.

Kindlin-1 is engaged in integrin β1 adhesion complexes, the focal adhesions, and regulates β1 activation, dynamics, and adhesion turnover (Harburger et al., 2009; Margadant et al., 2012). Besides, it acts as a linker between cell adhesion and regulation of the cell cycle (Patel et al., 2013) and controls Wnt and transforming growth factor-β availability to regulate stem cell proliferation (Rognoni et al., 2014).

Abbreviations: bp, base pair; EB, epidermolysis bullosa; FERMT1, gene coding for kindlin-1; KS, Kindler syndrome

Accepted article preview online 17 June 2015; published online 16 July 2015