Stearoyl-CoA Desaturase 1 Is a Novel Molecular Therapeutic Target for Clear Cell Renal Cell Carcinoma

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Abstract

**Purpose:** We set out to identify Stearoyl-CoA desaturase 1 (SCD1) as a novel molecular target in clear cell renal cell carcinoma (ccRCC) and examine its role in tumor cell growth and viability in vitro and in vivo independently as well as in combination with current U.S. Food and Drug Administration (FDA)-approved regimens.

**Experimental Design:** Patient normal and ccRCC tissue samples and cell lines were examined for SCD1 expression. Genetic knockdown models and targeted inhibition of SCD1 through use of a small molecule inhibitor, A939572, were analyzed for growth, apoptosis, and alterations in gene expression using gene array analysis. Therapeutic models of synergy were evaluated utilizing pharmacologic inhibition of SCD1 with the tyrosine kinase inhibitors (TKI) sunitinib and pazopanib, and the mTOR inhibitor temsirolimus.

**Results:** Our studies identify increased SCD1 expression in all stages of ccRCC. Both genetic knockdown and pharmacologic inhibition of SCD1 decreased tumor cell proliferation and induced apoptosis in vitro and in vivo. Upon gene array, quantitative real-time PCR, and protein analysis of A939572-treated or SCD1 lentiviral knockdown samples, induction of endoplasmic reticulum stress response signaling was observed, providing mechanistic insight for SCD1 activity in ccRCC. Furthermore, combinatorial application of A939572 with temsirolimus synergistically inhibited tumor growth in vitro and in vivo.

**Conclusions:** Increased SCD1 expression supports ccRCC viability and therefore we propose it as a novel molecular target for therapy either independently or in combination with an mTOR inhibitor for patients whose disease cannot be remedied with surgical intervention, such as in cases of advanced or metastatic disease. Clin Cancer Res; 19(9); 2368–80. ©2013 AACR.

Introduction

Currently clear cell renal cell carcinoma (ccRCC) is the most common subtype of RCC, which is the most prevalent kidney cancer among adults, accounting for approximately 89% of all diagnosed cases. Incidence of RCC has been increasing steadily in the United States, although mortality rates seem to have stabilized, likely because of early diagnosis among patients (1). Average age of diagnosis occurs typically between 60 and 64 years of age, although cases have been reported in younger individuals. Localized disease yields better patient outcomes as these cases can be addressed by partial or whole nephrectomy resulting in extended disease-free survival with 90% of stage I and 51% of stage II patients having an estimated 5-year or greater survival rate (2). For those patients diagnosed with advanced or metastatic disease the prognosis is markedly worse, with median survival periods dropping to approximately 3.1 and 1.1 years for stages III and IV patients, respectively (2). For individuals presenting with advanced disease, treatment options are limited with no current drug therapy leading to long-term survival (3) with the exception of 6% to 7% of patients who respond to interleukin-2 (4).

U.S. Food and Drug Administration (FDA) approved options for treatment of advanced ccRCC includes cytokine immunotherapy (interleukin-2, interferon; 4, 5) as well as treatments involving agents that target pathways believed to facilitate ccRCC progression such as angiogenesis and mTOR signaling (6). TKIs such as sunitinib (Sutent; ref. 7), pazopanib (Votrient; ref. 8), sorafenib ( Nexavar; ref. 9), and axitinib (Inlyta; ref. 10) block angiogenesis, through inhibition of VEGFR and PDGFR. Although there are treatments available for advanced ccRCC, none are curative and drug resistance occurs in all cases, often manifesting
Translational Relevance
Presently, there is a paucity of effective therapies designed to target clear cell renal cell carcinoma (ccRCC) effectuating long-term durable response in patients with advanced disease. In addition, there is a lack of molecular markers that can be remediably targeted, showing tumor-specific inhibition. Therefore, current therapeutic approaches often produce adverse side effects. We examined matched diseased and normal gene expression signatures, and identified that Stearoyl-CoA desaturase 1 (SCD1) was consistently overexpressed in ccRCC. Investigation of SCD1 as a molecular target for ccRCC intervention yielded antitumor activity in vitro and in vivo via activation of endoplasmic reticulum stress. Accordingly, induced endoplasmic reticulum stress proteins may be reliable biomarkers for response to therapy of an SCD1 inhibitor. Moreover, we discovered antitumor synergy with the FDA-approved mTOR inhibitor temsirolimus. This regimen may be a reasonable combinatorial therapy leading to enhanced efficacy and survival over that of current therapies for metastatic ccRCC.

SCD1 in ccRCC

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Materials and Methods

Chemicals
Temsirolimus, pazopanib, and sunitinib were purchased from Selleck Chemicals Co. Ltd. A939572 was purchased from BioFine International.

Cell culture
ccRCC cell lines: RWV366T and KI1265T (ref. 18; both stage IV ccRCC patient tissue derived; Copland Laboratory, Mayo Clinic, FL), A498, Caki1, Caki2, and ACHN (American Type Culture Collection, Manassas, VA) and K347N, K355N, K359N, K360N, K365N, and K366N normal renal tissue derived mortal cells (NRE) were prepared in dimethyl sulfoxide (DMSO; Sigma). Temsirolimus, pazopanib, and sunitinib were purchased from Selleck Chemicals Co. Ltd. A939572 was purchased from BioFine International.

Growth assays
Cells were plated (0.5 or 1 × 10^5/well) in 24-well plates (Midwest Scientific) in triplicate. Cells were counted using a Coulter Particle Counter (Beckman). Oleic acid–albumin (Sigma Aldrich) was added to media at 5 μmol. Drug stocks were prepared in dimethyl sulfoxide (DMSO; Sigma). Temsirolimus dosing was done as described in the text. Soft agar
cultures were prepared by diluting 2 × growth medium 1:1 in 1.5% SeaplaqueGTG agarose (Lonza), with 500 cells/plate in 60-mm culture dishes (Genesee Scientific). Colonies were stained with Giemsa (LabChem Inc.) and counted after 3 weeks.

**Lentivirus**

MISSION shRNA pLKO.1 constructs (Sigma-Aldrich) were used to make self-inactivating shRNA lentiviruses for human SCD1 (clones: NM_005063.3-1200s1c1[shSCD1200], NM_005063.3-780s1c1[shSCD780]), human ATF6 (NM_007348.1-332s1c1[shATF6-332]), and a nontarget random scrambled sequence control (SHC002). Transfection reagents Lipofectamine 2000 (Invitrogen) and ViraPower (Invitrogen) were used to generate lentiviruses using HEK293FT viral progenitor cells (Invitrogen). ccRCC and NRE cells were incubated with lentivirus plus 5 μg/mL polybrene (American Bioanalytical) for 24 hours before clonal selection with Puromycin (Fischer).

**Transfections and luciferase assays**

Caki1 and A498 cells were transiently transfected with p5xATF6-GL3 unfolded protein response (UPR) luciferase reporter [Addgene (plasmid#11976)] and pRL-CMV-renilla luciferase plasmid (Promega) using Lipofectamine2000 (Invitrogen). Cells (DMSO vs. A939572, nontarget vs. shSCD1200) were harvested after 48 hours using Promega’s Dual Luciferase assay kit per the manufacturer’s protocol and luciferase activity was measured using a Veritas Luminometer (Promega); reported as relative luminescence.

**RNA isolation and quantitative PCR**

RNA isolation, preparation of cDNA, and QPCR was conducted as previously described (14). TaqManFAM dye-labeled probes including POLR2A (Hs00172187_m1-normalization control), SCD1(Hs01682761_m1), HSPA5 (Hs99999174_m1). CEBPF(CEBPB Hs00270923_s1), GADD45A(Hs00169255_m1), Ddit3(Hs01090850_m1), Herpud1(Hs01124269_m1), and Atf6(Hs00232586_m1). Fold change value comparisons: normal versus tumor, nontarget versus target lentivirus, and DMSO versus A939572-treated samples using the ΔΔCt method (19).

**Gene array expression analysis**

Mayo Clinic Advanced Genomic Technology Center Gene Expression Core conducted gene array using Affymetrix Human Genome U133 Plus 2.0 Array chip. Data processing and methodology are as previously described (14). Gene expression data are deposited at Gene Expression Omnibus (Accession no. GSE41485). Pathway analysis was done using IPA (Ingenuity Systems).

**Western blot analysis**

Protein extraction and Western blot analysis was done as previously described (20). Primary antibodies included SCD1 (Sigma-Aldrich), PARP (Cell Signaling), Ddit3 (Cell Signaling), BIP (Cell Signaling), xBP1 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich).

**Immunohistochemical (IHC) and immunocytochemistry (ICC) analysis**

Samples include formalin-fixed, paraffin-embedded tissue microarray (TMA) of patient ccRCC tumor plus matched normal tissues and combinatorial in vivo mouse tumor tissue. Samples were mounted on slides, blocked with Diluent (Dakocytomation) for 30 minutes, and then probed as specified in text for SCD1, Ki67 (Invitrogen), Caspase-3 (Cell Signaling), CD31 (Santa Cruz Biotechnology), phospho-mTOR (Cell Signaling), Ddit3, and XBP1. ICC preparation and staining was done as previously described (18). Stain scoring was done using algorithms generated with Imagescope software (Aperio) created by a histologist. H-scores were calculated based upon signal intensity (0–3+) using the formula: [(1+9x1) + (2+9x2) + (3+9x3)], intensity (I)-scores were calculated by dividing signal intensity by area, and nuclear (N)-scores were calculated by dividing% positive nuclei by total nuclei examined per area. Cases where insufficient tumor tissue presented were excluded. 20× images were obtained using Scanscope XT and Imagescope software. This study was approved by the Mayo Institutional Review Board. RWV366T cell line validation was carried out as previously described (18).

**In vivo analysis**

A498 cells were subcutaneously implanted in athymic nu/nu mice (Harlan Laboratories) at 1 × 10⁶ cells/mouse in 50%Matrigel (BD Biosciences). Tumors reached ~50 mm³ before 4-week treatment. A939572 was resuspended in strawberry flavored Kool-Aid in sterilized H₂O (0.2 g/mL) vehicle at 30 mg/kg in a 50 μL dose. Mice were orally fed by using a syringe to administer the 50 μL dose twice daily/mouse. This modified method was found to be effective and less stressful on the mice. Temsirolimus was solubilized in 30% ethanol/saline and administered via intraperitoneal injection at 10 mg/kg in a 50 μL dose once every 72 hours/mouse. Tumor volumes were calculated using the formula 0.5236(L × W × H) and body weight were measured every 3 days.

**DNA isolation and STR analysis**

Genomic DNA was extracted from both RWV366T patient primary tissue and matching cell line using Purelink Genomic DNA mini kit (Invitrogen). Sixteen STR markers were PCR amplified using fluorescently labeled primers from ABI (Applied Biosystems), and were analyzed using ABI 3130 (Applied Biosystems). Peak sizes were calculated versus a coincjected size standard using Gene Marker (Soft Genetics).

**Statistical analysis**

Data values are presented as either percentage or fold change ± SD unless otherwise specified. Fold change values 1.5× are considered statistically significant. Treatment group comparisons were analyzed using 2-tailed paired Student t test with P < 0.05 being considered statistically significant. Statistically significant results are indicated by asterisk (*). Drug synergy statistics are indicated via
combination index (CI) determined using CalcuSyn (21) as described in the text.

Results

SCD1 is upregulated in clear cell renal cell carcinoma

Quantitative real-time PCR (QPCR) of total mRNA extracted from patient stage I to IV as well as metastatic ccRCC tumor tissues and matched distant site normal tissue was analyzed for gene expression of SCD1, and was found to be consistently upregulated in all tumor samples examined when compared to matched normal samples (Fig. 1A). Similarly, IHC staining for SCD1 protein expression in patient matched tumor and normal tissue confirmed elevated expression in tumor samples across all stages (Fig. 1B). Increased SCD1 expression in metastatic samples is due in part by induction of programmed cell death associated with loss of SCD1 and induction of cell death as a result of treatment, where OA-BSA supplementation with OA-BSA also decreased shSCD780 induced apoptosis confirmed by PARP cleavage shown by Western blot (Fig. 3B). In addition to growth rescue, supplementation with OA-BSA also decreased shSCD780 induced apoptosis as showed by reduction in PARP cleavage shown by Western blot (Fig. 3B). Representative phase contrast images of ccRCC cells for each group are shown in Fig. 3C.

Small molecule inhibition of SCD1 induces ccRCC cell death

A939572 is a small molecule that specifically inhibits SCD1 enzymatic activity (Supplementary Fig. 2A; ref. 22). A939572 showed a significant dose-dependent decrease in proliferation in Caki1, A498, Caki2, and ACHN at day 5 (IC50s of 65, 50, 65, and 6 nmol/L, respectively; Fig. 4A). Molecular target specificity was confirmed by addition of OA-BSA to the growth inhibitory assay, with IC50 doses applied to all 4 cell lines versus DMSO + BSA control. Addition of OA-BSA prevented A939572-mediated growth inhibition, comparable to control groups in all 4 cell lines (Fig. 4B). In congruity with previous experimentation examining SCD1 lentiviral knockdown models, A939572 induced apoptosis confirmed by PARP cleavage via Western blot analysis in all 4 cell lines (Fig. 4C). Addition of OA-BSA blocked apoptosis noted by lack of PARP cleavage (Fig. 4C). Representative phase contrast images (Fig. 4D) show marked reduction in confluence of A939572 treated ccRCC cells, which reflects decreased proliferation and induction of cell death as a result of treatment, where OA-BSA supplemented cells display no visible alterations in phenotype. Thus, we have identified a specific small molecule SCD1 inhibitor that induces apoptotic cell death that can be rescued by oleic acid.

Treatment of ccRCC cells with A939572 induces endoplasmic reticulum stress

To determine the mechanism of decreased proliferation and induction of cell death associated with loss of SCD1 activity in ccRCC cells, gene array analysis was conducted with Caki1, A498, Caki2, and ACHN ccRCC cells treated for 24 hours with a 75 nmol/L dose of A939572 compared to DMSO control. Gene expression data were analyzed using the Ingenuity Systems (IPA) program and revealed increased expression of endoplasmic reticulum stress markers in Supplementary Fig. S1A and S1B.

SCD1 in ccRCC

Oleic acid reverses effects of decreased SCD1 expression in tumor cells

As oleic acid (OA) is the principle product of SCD1-mediated SFA dehydrogenation (15), a cell culture stable form of OA conjugated to albumin from bovine serum (OA-BSA) was utilized to conduct rescue experimentation to confirm that decreased tumor cell growth and induction of cell death was because of lentiviral-mediated suppression of SCD1.

Media alone and BSA served as control groups. Proliferation assay of nontarget control versus shSCD780 infected Caki1 and A498 cells supplemented with or without OA-BSA were counted after 5 days. Both Caki1 and A498 shSCD780 cells exhibited significant decreases in growth when compared to controls; however, the addition of OA-BSA rescued the proliferative capacity of these cells to near control rates (Fig. 3A). Notably, addition of OA-BSA to Caki1 NT cells marginally enhanced proliferation (Fig. 3A). SCD1 knockdown by lentiviral infection was confirmed at the protein level (Fig. 3B). In addition to growth rescue, supplementation with OA-BSA also decreased shSCD780 induced apoptosis as showed by reduction in PARP cleavage shown by Western blot (Fig. 3B). Representative phase contrast images of ccRCC cells for each group are shown in Fig. 3C.

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SCD1 is required for ccRCC tumor cell growth and survival

To identify the role of increased SCD1 in regards to ccRCC viability as well as validate specificity in tumor compared to normal samples, 2 lentiviral constructs designed to target SCD1 (shSCD780 and shSCD1200) were used to knockdown SCD1 expression in 2 NRE (K359N, K360N) and 2 ccRCC cell lines (Caki1, A498). K360N was included as an NRE control as it showed protein expression of SCD1. QPCR analysis for SCD1 expression confirmed over 80% mRNA silencing in all samples relative to nontarget controls for each cell line (Fig. 2A). Specificity of shSCD780 and shSCD1200 for SCD1 was confirmed by Western blot (Fig. 2B). Subsequent growth analysis resulted in over an 80% decrease in proliferation among tumor samples at day 5 postinfection (Fig. 2B) but not in NRE samples (Fig. 2C). Because such a considerable proliferative decrease in tumor cells was observed, Western blot analysis for poly-ADP-ribose polymerase (PARP) cleavage, a marker for apoptosis, was done. Results confirmed PARP cleavage in both Caki1 and A498 cells infected with each shSCD lentiviral construct compared to NT controls (Fig. 2D), and indicates that loss of proliferation is due in part by induction of programmed cell death.

Oleic acid reverses effects of decreased SCD1 expression in tumor cells

As oleic acid (OA) is the principle product of SCD1-mediated SFA dehydrogenation (15), a cell culture stable
Figure 1. SCD1 expression is upregulated in ccRCC. A, SCD1 mRNA expression shown for ccRCC tissue samples relative to matched normal samples across stages I to IV with average fold change increases of 29.7 (n = 7), 18.0 (n = 8), 30.0 (n = 8), 23.9 (n = 12), and 11.5 (n = 8) in stage 1 to 4 and metastatic groups respectively, graphed as a box and whisker plot. B, IHC for SCD1 expression in patient normal and matched ccRCC tissue stage I to IV with average H-score expression ranging from 134.7 to 157.9 in tumor versus 22.6 to 37.0 in normal (n = 42, 24, 35, 11 in tumor stages 1–4 and 40, 30, 37, 5 for normal matches, respectively). Western blot analysis and quantitation of SCD1 expression in protein lysates prepared from patient metastatic tissue matched to distant site normal samples. C, mRNA expression analysis of NRE versus ccRCC cell lines with fold change inductions of 28.4, 14.4, 17.0, 4.0, 38.6, and 12.4 seen in Caki1, Caki2, A498, ACHN, KIJ265T, and RWV366T when normalized to 366N, which possessed the lowest SCD1 expression and is set at 1. SCD1 expression in NREs ranged from 1 (in 366N) to 3.2 (in 365N). D, Western blot for SCD1 protein expression in NRE and ccRCC cell lines. *, P < 0.05.
response genes associated with UPR (23). A summary of endoplasmic reticulum stress genes induced by A939572 treatment is listed along with median fold change induction in Supplementary Fig. S2B.

Western blot of Caki1 and A498 cells for protein expression of endoplasmic reticulum stress markers including BiP (heat shock 70 kDa protein, GRP78), CHOP (DNA damage inducible transcript 3, DDIT3), and spliced-XBP1 (x-box binding protein 1, s-XBP1) revealed amplified expression in both drug treated (75 nmol/L) and shSCD780 lentiviral knockdown cells after 48 hours (Fig. 5A), confirming induction of endoplasmic reticulum stress upon loss of SCD1 activity or expression as implicated by the gene array analysis.

To validate the specificity of endoplasmic reticulum stress induction mediated by both A939572 and shSCD780, rescue assays were done using OA-BSA in Caki1 and A498 cells. QPCR analysis of 5 endoplasmic reticulum stress genes identified in the gene array including BiP, CHOP, HERPUD1 (homocysteine-inducible, endoplasmic reticulum stress inducible, ubiquitin-like-1), GADD45a (DNA damage inducible transcript 1, DDI1), and CEBPβ (CCAAT/enhancer binding protein β) were examined. In A939572 (SCDi) treated Caki1 and A498 cells, all 5 endoplasmic reticulum stress related genes were expressed at significantly increased levels compared to DMSO + BSA control, and this elevated expression could be blocked with the addition of OA-BSA (Fig. 5B). In shSCD780 lentiviral infected Caki1 and A498 cells, all of the endoplasmic reticulum stress genes were significantly induced in the Caki1 shSCD780 sample and 4 of the 5 were significantly induced in the A498 shSCD780 sample. Similar to the drug-treated cells, OA-BSA successfully blocked shSCD780 induced expression of the endoplasmic reticulum stress genes (Fig. 5B).

Endoplasmic reticulum stress induction due to genetic and pharmacologic inhibition of SCD1 was assessed in NRE cells. Because A939572 did not yield a dose-response in NRE cells (Supplementary Fig. S2C), a dose of 100 nmol/L was applied for 48 hours. No significant endoplasmic reticulum stress induction, measured by QPCR for endoplasmic reticulum stress markers CHOP, HERPUD1, GADD45a, and BiP, was observed in K359N utilizing either lentiviral(shSCD780) or A939572 SCD1 inhibition.
inhibition (Supplementary Fig. S2D). Interestingly, lentiviral inhibition of SCD1 in K360N cells, which do show SCD1 protein expression (Fig. 1D), yielded no induction of endoplasmic reticulum stress, however A939572 treatment led to a slight induction of endoplasmic reticulum stress as 2 of the 4 endoplasmic reticulum stress markers evaluated, HERPUD1 and BiP, were upregulated ~2-fold (Supplementary Fig. S2D). Nevertheless, the endoplasmic reticulum stress induction observed in A939572-treated K360N is milder in comparison to QPCR values of endoplasmic reticulum stress induction observed in A498 and Caki1 ccRCC cells (Fig. 5B), and neither A939572 or lentiviral inhibition of SCD1 in K360N led to attenuated cell proliferation in K360N (Fig. 2C, Supplementary Fig. S2C). Thus, endoplasmic reticulum stress is strongly induced in SCD1 expressing ccRCC cell lines.

Activating transcription factor 6 (ATF6) is a key bZIP transcription factor that mediates part of the UPR stress response (24, 25). Upon stress induction, ATF6 incites activation of many downstream mediators in the endoplasmic reticulum stress response pathway including XBP1, BiP, HSP90B1 (heat shock protein 90 kDa beta), GADD45a, HERPUD1, and CHOP (25, 26). Caki1 and A498 cells transfected with an ATF6 luciferase reporter (p5xATF6-GL3; ref. 24) were treated with A939572 or were infected with shSCD780. Inhibition of SCD1 resulted in significant enhancement of luciferase activity as compared to DMSO and nontarget controls, where the addition of OA-BSA significantly reduced reporter activation (Supplementary Fig. S3). This confirms that ATF6 is activated by inhibition of SCD1 activity in ccRCC cell lines.

To deduce whether endoplasmic reticulum stress directly mediates the effects on proliferation and cell death observed exclusively in ccRCC cell lines due to SCD1 inhibition (Figs. 2-4), ATF6 was silenced using a lentiviral construct (shATF6-332) in Caki1 and A498 ccRCC cells. Effects on endoplasmic reticulum stress induction and proliferation were evaluated in the presence of A939572. QPCR for ATF6 expression exhibited attenuation of over 85% in both Caki1 and A498 shATF6-332 cells both in DMSO and A939572-treated groups when compared to NT DMSO controls (Fig. 5C). QPCR analysis for endoplasmic reticulum stress markers regulated by activation of ATF6 (CHOP, HERPUD1, and GADD45a) was done. Transcription expression levels displayed that knockdown of ATF6 blocked A939572-mediated induction of CHOP, HERPUD1, and GADD45a in both cell lines, and in fact decreased levels of HERPUD1 in Caki1 cells, and CHOP in both cell lines (Fig. 5C). This suggests that ATF6 knockdown successfully compromised the induction of the endoplasmic reticulum stress response in ccRCC. Proliferation assay of nontarget and shATF6-332 in DMSO versus A939572 showed that ATF6 suppression rescued decreased proliferation observed in nontarget samples due to treatment with A939572 in both Caki1 and A498 cells (Fig. 5D). This data supports that loss of ccRCC viability via inhibition of SCD1 is conducted through the endoplasmic reticulum stress response.

Combination of A939572 with temsirolimus synergistically enhances tumor cell death

To target ccRCC using a multifaceted approach, synergy was examined through application of combinatorial treatment utilizing A939572 in congruence with a current FDA approved regimen for ccRCC treatment. These included the TKIs pazopanib and sunitinib, as well as the mTOR inhibitor temsirolimus.

After identifying appropriate cell proliferative dose responses for pazopanib and sunitinib in 4 ccRCC cell lines including A498, Caki1, Caki2, and ACHN (Supplementary Fig. S4A and S4C), both TKIs were dosed in combination with A939572 (Fig. 3). Antiproliferative and apoptotic induction via loss of SCD1 expression can be rescued with addition of oleic acid (OA-BSA). A, proliferation and (B) Western blot analysis for SCD1 and PARP cleavage in Caki1 and A498 NT versus shSCD with or without OA-BSA supplementation. C, phase-contrast microscopy displays representative ccRCC cell (Caki1) confluence at day 5 of proliferation assay with different treatment conditions. *, P < 0.05.
with A939572 up to approximately the IC_{50} dose for each drug in the Caki1 and the A498 cell lines. No synergy was noted in either Caki1 or A498 cells with combinatorial treatment (Supplementary Fig. S4B and S4D). No dose–response could be determined for temsirolimus (Tem; Supplementary Fig. S5). Combinatorial treatments were therefore done using a fixed dose of Tem (0.1, 1, and 10 nmol/L) combined with a dose range of A939572 up to the IC_{50} in Caki1, A498, Caki2, and ACHN cells. Both drugs in combination yielded very strong synergy in all 4 cell lines (Supplementary Fig. S6A–S6D) as indicated by the CI determined using CalcuSyn (21) based on the Chou–Talalay method where CI values >1 represent an antagonistic effect and values <1 represent synergy, with lower values signifying enhanced synergy (27). Colony formation assay of A498 cells grown in soft agar treated with mono and combination doses of 5 nmol/L A939572 and 5 nmol/L Tem (Supplementary Fig. S5) reflected synergistic effects observed in combination proliferation assays, and provided the rationale for in vivo analysis of combinatorial therapy.

Athymic nude (nu/nu) mice bearing A498 ccRCC xeno-grafts were treated with A939572 and Tem individually or in combination over the course of 4 weeks, and tumor volume (mm³) was recorded (Fig. 6A). A939572 and Tem mono-therapy generated similar growth responses with approximately 20% to 30% reductions in tumor volume (vs. placebo control) being observed upon study completion, with values reaching statistical significance only within the last week of treatment. The combination group yielded over a 60% decrease in tumor volume (vs. placebo control) by study completion with significant reductions recorded after approximately 1 week of treatment. All of the animals maintained a healthy weight throughout the course of the treatment (Fig. 6A), however those in both the A939572 and the Combo group exhibited increased blinking, and slight mucosal discharge from the eyes after the first week of treatment.

IHC analysis of tumors resected from each treatment group was analyzed for proliferation, angiogenesis, and cell death (Fig. 6B). All treatment groups (A939572, Tem, and Combo) when compared to the placebo control exhibited decreased proliferation as marked by reduction in percent positivity of nuclear Ki67 staining, with the combinatorial group showing the most significant decline. Angiogenesis as examined by intensity of microvessel density showed a slight decrease in both the Tem and the Combo groups;
Figure 5. Inhibition of SCD1 activity in ccRCC induces cell death mediated by endoplasmic reticulum stress response. A, Western blot analysis for expression of endoplasmic reticulum stress markers BiP, CHOP, and spliced XBP1 in response to A939572 treatment or lentiviral silencing of SCD1 in Caki1 and A498. B, QPCR analysis of endoplasmic reticulum stress gene expression in Caki1 and A498 cells treated with A939572 (75 nmol/L) or shSCD1 lentivirus ± OA-BSA rescue after 48 hours. C, QPCR for ATF6 expression in nontarget and ATF6 knockdown (shATF6-332) Caki1 and A498 cells treated with DMSO or IC50 dose of A939572 (SCDi) for 48 hours (far left). QPCR for endoplasmic reticulum stress markers CHOP, HERPUD1, and GADD45a in Caki1 (middle) and A498 (far right) ccRCC cells treated with A939572 (IC50 dose for 48 hours); expression is normalized to nontarget DMSO sample for all groups. Statistically significant correlations between DMSO and SCDi-treated nontarget or shATF6-332 DMSO and shATF6-332 SCDi are denoted by single asterisk (*, P < 0.05.). Statistically significant correlations between nontarget DMSO and each shATF6-332 DMSO and shATF6-332 SCDi are denoted by single asterisk (*, P < 0.05.). D, proliferation of nontarget and shATF6-332 Caki1 and A498 RCC cells treated with DMSO control or A939572. Doses utilized are as described in C, and cells were counted 72 hours posttreatment.
Figure 6. Treatment of ccRCC cells with SCD1 inhibitor in combination with the mTOR inhibitor temsirolimus synergistically inhibits tumor cell growth in vivo. A, in vivo tumor growth analysis and animal weight of A498 ccRCC subcutaneous xenografts in female athymic nude mice treated with A939572 and temsirolimus alone or in combination versus placebo control (n = 10 per group). B, IHC of tissue harvested from treatment groups stained for Ki67 (quantitated by N-score), CD31 (quantitated by I-score), and phospho-mTOR (quantitated by H-score). Average group scores ± SE are reported for each stain. C, Western blot and quantitation of CHOP expression in all 4 treatment groups. D, model of proposed SCD1 activity in ccRCC model: Inhibition of SCD1 blocks desaturation of SFA resulting in an accumulation of SFA species, which trigger the endoplasmic reticulum stress response.
however, the cumulative scores were not considered significant. Cell death as examined by cleaved caspase-3 (CC3) showed significant increases in the Combo group when compared to all groups. A moderate increase in cell death was also seen in the A939572 and Tem groups compared to the placebo. Phosphorylated mTOR was inspected as a marker for temsirolimus activity, and decreased expression was confirmed in both the Tem and the Combo groups as compared to the Placebo group. Endoplasmic reticulum stress was examined via Western blot of total protein extractions prepared from randomly selected tumor tissue samples representing each treatment group, and resulting quantitative expression was normalized to respective β-actin controls. Increased expression of CHOP was confirmed in all samples treated with A939572 (A939572 and Combo; Fig. 6C), confirming that inhibition of SCD1 in ccRCC contributes to endoplasmic reticulum stress in vivo. Interestingly, samples in the Tem group also exhibited induction of CHOP, although to a lesser extent when compared to A939572 and Combo groups. Temsirolimus has been previously reported to decrease SCD1 expression in breast cancer cells (28). Inhibition of mTOR in ccRCC could indirectly mediate endoplasmic reticulum stress through decrease of SCD1, thereby explaining our observations. Little to no CHOP expression was seen in any placebo samples. In summary of our cumulative findings, we propose that attenuation of SCD1 activity by either genetic knockdown or small molecule inhibitors promotes endoplasmic reticulum stress through accumulation of SFA species as depicted in Fig. 6D via a mechanism that is conversed in detail in the following discussion.

Discussion

Despite current advances in cancer research, which have improved our understanding of cancer cell biology, there are still limitations in current therapeutic options for patients with advanced ccRCC (3, 6). Two primary explanations for this include the lack of molecular targets in ccRCC, which manifest consistently in a significant proportion of patient tumor samples. Also, the adaptive nature of tumor cells typically results in acquired drug resistance with selective pressure sometimes yielding outgrowth of more malignant subpopulations (29–31). It is clear that development of more effective therapeutic strategies must encompass identification of molecular targets as well as employing multitargeted approaches to minimize disease recurrence.

In ccRCC, de novo lipogenesis is highly activated compared to normal cells (14, 32). Of the lipid metabolic genes deregulated in ccRCC, we found SCD1 expression to be consistently amplified in nearly all patient samples across all stages of disease. Selective targeting of SCD1 decreased tumor cell proliferative capacity and promoted apoptosis while producing no notable effects in NRE cells, thereby making SCD1 an ideal candidate for therapeutic intervention in patients presenting with early and late-stage ccRCC. In addition, SCD1 itself may serve as a predictive biomarker, where positive SCD1 IHC would identify appropriate can-

didate patients who would likely yield a response to pharmacological inhibition of SCD1. SCD1 expression has been shown to be upregulated in numerous neoplastic lesions including lung adenocarcinoma, non–small cell lung, hypopharyngeal, gastric, breast, prostate, ovary, and colon carcinoma (33–36). Previous experimentation has produced promising results in numerous in vitro and in one preclinical experiment showing reduction of tumor volume in a gastric carcinoma model in response to small molecule inhibition of SCD1 (34). SCD1 has therefore been proposed as a molecular target for multiple tumor types, whose list now includes ccRCC. Although SCD1 expression in normal cells seems to be well characterized, how tumor cells override conventional regulations to induce its expression still remains to be clarified.

Mechanistically, inhibition of SCD1 activity revealed increased expression of endoplasmic reticulum stress markers consistent with the activation of the UPR pathway, suggesting that SCD1 activity positively regulates endoplasmic reticulum homeostasis. These results are consistent with previous findings in an in vitro setting (34, 37), and our group has now confirmed this in vivo with our ccRCC tumor xenograft model. In addition, our work mechanistically links endoplasmic reticulum stress induction due to SCD1 inhibition as the mediator of apoptosis, and may therefore serve as a practical biomarker for response to anti-SCD1 therapy. The UPR is characterized by signaling through core endoplasmic reticulum stress sensors ATF6, PERK (PKR-like endoplasmic reticulum kinase), and IRE1 (Inositol-requiring enzyme-1; ref. 38). These central signal transducers regulate several responses downstream including: transcription of molecular chaperones, ubiquitin ligase activity, attenuation of translation, as well as activation of other protective or proapoptotic factors. These reactions all result in either the restoration of cellular homeostasis or cell death in prolonged or severe cases of stress (39, 40). Previous studies have linked SFA accumulation to induction of endoplasmic reticulum stress (41, 42), and we believe that loss of SCD1 activity plays a role in this process, although the mechanism of de novo lipid biosynthesis in conjunction with endoplasmic reticulum stress needs to be further investigated. Current literature suggests that increased exposure of cells to SFAs corresponds to an accumulation of SFA content in membrane structures, altering the morphology and decreasing membrane fluidity (43). This may compromise the integrity as well as the functionality of the membranes, including those of the endoplasmic reticulum, leading to a stress response (43–45). Desaturation of fatty acids is thought to counter these effects, and is protective against SFA-mediated stress (43, 46, 47). This proposed mechanism is summarized in Fig. 6D.

To address the notion of utilizing multitargeted therapy as a more effective strategy for treatment of ccRCC, combinatorial options were examined pairing A939572 with current standard-of-care regimens. Previous experimentation targeting SCD1 pharmacologically has showed
minimal adverse side effects, and this suggests a reduced risk of compounding drug toxicity when used in combinatorial therapy. Those reported using animal models include dry eye, squinting, and alopecia; which proved to be reversible upon discontinuation of treatment (48, 49). Observations from our own in vivo study using an SCD1 inhibitor corroborate these findings. Of note, given the role of SCD1 in lipid metabolism, it is currently being investigated as an attractive target for the treatment of diabetes, obesity, and other metabolic diseases; however, detailed effects of SCD1 inhibition on metabolism as well as toxic effects in humans remain to be completely defined (48, 50). One clinical trial investigating the use of a liver-targeting SCD1 inhibitor MK-8245 (48) for treatment of type II diabetes produced no serious adverse events in all patients evaluated (clinical trial identifier NCT00790556). Our investigations identified a robust synergistic combinatorial response in ccRCC cells both in vitro and in vivo to A939572 paired with temsirolimus. The exact mechanism of this synergistic collaboration is not well defined, and further studies must be conducted. Also, although we did not observe synergistic inhibition of tumor cell growth utilizing A939572 in combination with approved TKIs, we believe that this course of therapy warrants deeper investigation because of the limitations of examining drug effects in vitro versus that of animal models where angiogenesis plays key role in tumor growth.

In conclusion our findings support that increased SCD1 expression is tumor specific in ccRCC, and is maintained throughout the course of disease progression. Genetic and molecular targeting of SCD1 activity results in tumor-specific inhibition of cell growth and induction of apoptosis both in vitro and in vivo, which is mediated by the endoplasmic reticulum stress response. Finally, combined treatment of ccRCC with A939572 and an mTOR inhibitor resulted in synergistic inhibition of tumor growth. Taken together, we propose SCD1 as a novel molecular target for the treatment of ccRCC, which should be investigated as a therapeutic option alone or in combination with mTOR inhibitors for patients suffering from advanced or metastatic ccRCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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