Quantitative proteomics to study a small molecule targeting the loss of von Hippel–Lindau in renal cell carcinomas

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Inactivation of the tumor suppressor gene, von Hippel–Lindau (VHL), is known to play an important role in the development of sporadic clear cell renal cell carcinomas (ccRCCs). Even if available targeted therapies for metastatic RCCs (mRCCs) have helped to improve progression-free survival rates, they have no durable clinical response. We have previously shown the feasibility of specifically targeting the loss of VHL with the identification of a small molecule, STF-62247. Understanding its functionality is crucial for developing durable personalized therapeutic agents differing from those available targeting hypoxia inducible factor (HIF) pathways. By using SILAC proteomics, we identified 755 deregulated proteins in response to STF-62247 that were further analyzed by Ingenuity pathway analysis (IPA). Bioinformatics analyses predicted alterations in 37 signaling pathways in VHL-null cells in response to treatment. Validation of some altered pathways shows that STF-62247’s selectivity is linked to an important inhibition of mTORC1 activation in VHL-null cells leading to protein synthesis arrest, a mechanism differing from two allosteric inhibitors Rapamycin and Everolimus. Altogether, our study identified signaling cascades driving STF-62247 response and brings further knowledge for this molecule that shows selectivity for the loss of VHL. The use of a global SILAC approach was successful in identifying novel affected signaling pathways that could be exploited for the development of new personalized therapeutic strategies to target VHL-inactivated RCCs.

Kidney cancer is the ninth most diagnosed cancer in the world.1,2 ccRCC, the most frequent kidney cancer, is associated with biallelic inactivation of the tumor suppressor gene VHL.3,4 Multiregion exome sequencing studies indicated an elevated intratumor heterogeneity in sporadic ccRCCs and showed that VHL inactivation was the only truncal mutation present in all cases.5 The most characterized function for VHL protein (pVHL) is as a recognition component of an E3 ubiquitin–ligase complex (VCB-Cul2 complex) that targets hypoxia inducible factors alpha (HIFα) for ubiquitination and consequent proteasomal degradation.6–9 Comprehension of the VHL-HIF axis brought to light the development of targeted therapies for advanced ccRCC but is unfortunately of limited efficacy and carries significant toxicity.9 Thus, the authors declare no potential conflicts of interest.

Key words: renal cell carcinoma, von Hippel–Lindau, autophagy, mTOR, protein synthesis, targeted therapy, cell death

Abbreviations: ATG: autophagy related genes; ccRCCs: clear cell renal cell carcinomas; CHX: cycloheximide; DAVID: database for annotation, visualization and integrated discovery; DMEM: Dulbecco’s modified Eagle medium; FBS: fetal bovine serum; FP: free-pool; GO: gene ontology; HG: high glucose; HCL: hydrochloric acid; HIF: hypoxia inducible factor; HIFα: hypoxia inducible factors alpha; IPA: ingenuity pathway analysis; IAA: iodoacetic acid; mTOR: mammalian target of Rapamycin; MSD: mass selective detector; MS: mass spectrometry; mRCCs: metastatic RCCs; NT: nontargeting; PCA: principal component analysis; ROS: reactive oxygen species; SOM: self-organizing maps; STR: short tandem repeat; SILAC: stable isotope labeling by amino acids in cell culture; pVHL: VHL protein; VHL: von Hippel–Lindau

The authors declare no potential conflicts of interest.

Significance: Mutations in VHL gene are reported in most RCC cases. We previously demonstrated the possibility to target VHL-deficient cells using small molecules. Our study identified a list of 755 deregulated proteins in response to STF-62247 by quantitative proteomics. Validation of predicted altered pathways shows that the STF-62247 is associated with a selective mTOR inhibition and arrest of protein synthesis in VHL-mutated cells. Findings from this work contribute to our understanding of the small molecule.

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What's new?
Mutations on the tumor suppressor gene VHL are observed in most RCC cases. We previously identified a small molecule, STF-62247 that is capable to kill VHL-inactivated tumor cells. Here they used SILAC proteomics, IPA, and bioinformatics to show that STF-62247 may arrest protein synthesis in VHL-negative cells, while sparing normal cells. This approach also identified novel signaling pathways that could provide new, personalized therapeutic targets for ccRCC.

Material and Methods
Mammalian cell culture and treatments
RCC4 cells were cultured in DMEM-HG supplemented with 50 mg L-Lysine-2HCl and 50 mg L-Arginine-HCl (light) or with 50 mg 15C6 L-Lysine-2HCl and 50 mg of 13C15N2 L-Arginine-HCl (heavy) (Cambridge Isotope Labeling, Tewksbury, MA). 3.5 × 10⁵ cells were cultured in parallel in heavy or light media for 7 days and were lysed with the Mammalian-Protein Extraction Buffer (M-PER) lysis buffer. In-gel digestion of proteins was performed, offering better resolution and increasing the number and accuracy of proteins present in the samples. Briefly, proteins from both conditions were mixed in different ratio quantities and separated by SDS-PAGE on a 10% bis-acrylamide gel. The gel was stained with EZ Blue Gel Staining Reagent (Sigma–Aldrich Canada Co., ON, Canada) and bands were excised and digested with Trypsin for Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) analysis. Each lane was cut in 24 gel pieces, washed and destained with acetonitrile. Dithiothreitol (DTT) (10 mM) and iodoacetic acid (IAA) (25 mM) solutions were used for reduction and alkylation of protein bands, respectively. 20 ng/mL of Trypsin Gold (V5280, Promega, Madison, WI) was added to the gel pieces and incubated overnight at 37°C. Extraction of peptides was achieved by adding 5% acetic acid in 50% acetonitrile. Heavy or light (INV)-labeled RCC4 cells were treated with 1.25 μM STF-62247 for 48 hr.

Mass spectrometry analysis
Protein digests were analyzed by gradient nanoLC-MS/MS using a Quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Scientific, San Jose, CA) interfaced to a Proxeon Easy Nano-LC (Thermo Scientific). High-resolution chromatographic separation was achieved on an Easy C18 analytical column with dimensions of 100 mm by 75 μm i.d. using 3 μm diameter ReproSil-Pur particles. Peptide elution was achieved using an acetonitrile/water gradient system. Solvent A was 0.1% formic acid (Sigma–Aldrich) in water and solvent B was acetonitrile/water/formic acid (90/9.9/0.1). A linear acetonitrile gradient was applied to the C18 column from
5 to 30% solvent B in 60 min followed by 100% B for 10 min at a flow rate of 300 nL/min. Precursor ion spectra were collected at a resolution setting of 35,000 and an automatic gain control value of $1 \times 10^5$. Peptide fragmentation was performed using high-energy collision-induced dissociation in the HCD cell and MS/MS spectra were collected in the orbitrap at a resolution of 17,500 and an AGC setting of $1 \times 10^5$. Peptide precursors were selected using a repeat count of 2 and a dynamic exclusion period of 20 sec. Protein identification and quantification data were analyzed using Proteome Discoverer version 1.4 (Thermo Scientific) employing the Sequest scoring algorithm. FASTA databases were obtained from Uniprot for *Homo Sapiens*. Quantification of 2-plex SILAC data was performed by Proteome Discoverer Precursor Ions Quantifier node using a heavy labeled arginine (+10,008 Da) and lysine (+6,020 Da) with a minimum fold change of 2 to indicate a significant result. Scaffold Q+ (version Scaffold_4.3.4, Proteome Software, Inc., Portland, OR) was used to validate SILAC peptide and protein identifications and quantitation. Acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run.

**Microarray hybridization and analysis**

Quality of total RNA samples was assessed using the Experion bioanalyzer system with RNA StdSens chips and associated reagents (Bio-Rad, ON, Canada). One microgram of total RNA samples was amplified using the Amino Allyl MessageAmp II aRNA amplification kit and subsequently labeled with AlexaFluor 555 or 647 (Thermo Scientific). Samples were compared in a dye swap experiment, with 2.5 μg of each labeled, fragmented aRNA (5 μg total per slide) hybridized to proprietary human cDNA microarray slides. These arrays consist of approximately 35,000 spots, representing roughly 17,000 different 50-mer oligonucleotides spotted in duplicate on Nexterion-E epoxy microarray slides (Schott). Hybridizations were performed in Ambion SlideHyb #2 buffer (Thermo Scientific) at 42°C for 16 hr using the automated TECAN 4800 Hybridization station (TECAN). Analysis was done with Acuity 4.0 (Axon Instruments). The data was normalized using Lowess and analyzed by various statistical methods such as SOM (Self Organizing Maps), t test, PCA (principal component analysis) and volcano plot.

**Bioinformatics and data analysis**

Differentially expressed proteins were analyzed using IPA (Qiagen Ingenuity systems, Redwood City, CA). Functional networks, canonical pathways and molecular functions were generated based on information contained in the ingenuity pathways knowledge base. The database for annotation, visualization and integrated discovery (DAVID) was used as a web-based online bioinformatics resource for a functional interpretation of differentially expressed proteins identified by SILAC.

**Western blot**

Cells were lysed in M-PER buffer and quantified using Pierce BCA protein assay Kit. Immunoblots were probed with the following antibodies: β-Actin, α-Tubulin, UBA1, 14–3–3ε, Cathepsin D, p62/SQSTM1 (Santa Cruz Biotechnologies, Santa Cruz, CA), TMEM59 (Novus Biologicals, ON, Canada), mTOR, p-mTOR (S2448), p70S6K, p-p70S6K (T389), p-4EBP1 (T37/46), Rab5 and Rab7 (Cell Signaling Technologies, Danvers, MA). Chemiluminescence detection was performed using the ECL detection system and molecular imager system (Chemi-Doc XRS system, BIO-RAD, Inc., ON, Canada).

**Measurement of reactive oxygen species (ROS)**

Intracellular ROS production was assessed using 2′,7′-dichlorodihydrofluorescein (H2DCFDA) probe (Life technologies, Thermo-Fisher). Cells were harvested and incubated with 10 μM H2DCFDA for 30 min at 37°C. Fluorescence was measured using excitation at 485 nm and emission at 530 nm. Relative quantification was calculated between treated and untreated cells.

**Transient transfection**

Cells were transfected using validated siGENOME Human siRNA targeting EEF2 (SMARTpool) and siControl2 nontargeting pool from Dharmacon using Dharmafect reagent 1 for 72 hr. Cells were trypsinized for clonogenic assays and proteins were extracted for western blot.

**XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide) and clonogenic assays**

**XTT assays.** Five-thousand cells were plated per well (96-well plate) and treated with serial dilutions of STF-62247, RAD001 or Rapamycin. Combinations of 1.25 μM STF-62247 and serial dilution of RAD001 or Rapamycin were also plated. After 4 days, XTT solution (0.3 mg/mL of XTT (Sigma–Aldrich), phenol red-free media, 20% FBS and 2.65 μg/mL N-methyl dibenzopyrazine methyl sulfate [PMS]) was added to cells and incubated at 37°C for 1 hr. Absorbance was read at 450 nm on a Spectramax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

**Clonogenic assays.** Five-hundred cells were plated in triplicate in 60 mm plates and treated 24 hr later with 1.25 μM STF-62247 and left at 37°C for 8 days. Plates were stained with a solution of crystal violet and colony formation was quantified.

**Protein synthesis measurement**

First, protein synthesis was determined by Click-IT HPG AlexaFluor protein Synthesis Assay Kit (Thermo Fisher) and performed per manufacturer’s instructions. Briefly, cells were plated in an eight-well chambered coverglass (LabTek) and treated with STF-62247. Click-IT HPG solution (in L-methionine free media) was added for 30 min, fixed with
3.7% formaldehyde and permeabilized with 0.5% Triton X-100. Click-iT HPG detection was achieved by adding reaction cocktail for 30 min at RT and rinsed with the reaction buffer. DNA staining with HCS NuclearMask Blue Stain was incubated for 30 min. Images were acquired with an Olympus Fluoview FV1000 confocal microscope.

Second, a protocol for measuring the fractional rate of protein synthesis described by S.G. Lamarre’s group was adapted for mammalian cells. To establish the optimal incorporation time of ring-D$_3$ L-phenylalanine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) in cells, 300 µL of a 150 mM solution of phenylalanine and phenylalanine-D$_3$ (50:50) prepared in water was added to RCC4 and RCC4 VHL cells for 0, 15, 30, 45 and 60 min. One milliliter of media from each dish was kept and ice-cold perchloric acid was added (free-pool [FP] of amino acids). Cells were scrapped directly in 1 mL of 0.2 M ice cold perchloric acid and left on ice for 10 min. FP and cell fractions were centrifuged 15,000 g, 5 min at 4°C. Supernatants were discarded and protein pellets were resuspended in 0.2 M perchloric acid and centrifuged for 15,000 g, 10 min. After repeating this step three times, the pellet is washed with acetone and centrifuged before being hydrolyzed in 6 mL 6 N hydrochloric acid (HCL) at 110°C overnight. Phenylalanine extraction and derivatization was done as per S.G. Lamarre et al. (2015). The determination of PHE specific enrichment was determined using an Agilent gas chromatograph (model 6890N) interfaced with a single quadrupole inert mass selective detector (MSD, model 5973).

**Results**

**SILAC experimental procedures and data compilation**

Our optimized SILAC workflow consists of two main steps; (i) determination of isotope incorporation efficiency (Fig. 1a, left) and (ii) SILAC main experiment/treatment (Fig. 1a, right). First, VHL-deficient RCC4 cells were grown in parallel in light or heavy isotope containing-medium for five cell doublings and both protein populations were extracted and quantified (Fig. 1a, left). To ensure complete (>95%) incorporation efficacy and standard quantification, heavy and light samples were loaded separately on a polyacrylamide gel and different ratios of each were mixed (Heavy/Light; 1:1, 1:2, 1:5, 2:1, 5:1). A band with the same molecular mass was excised in each lane, trypsin digested and processed by LC-MS/MS to validate isotope incorporation ratios. Heavy isotope peptides were identified by a shift of 6 and 10 Da as expected by the presence of Lysine and Arginine (Fig. 1a, left). Secondly, cells in Heavy-medium were treated for 48 hr with STF-62247. Proteins from heavy- and light-untreated cells were separated on gel. “INVERT SILAC” (INV) was utilized as a control by treating cells in light-medium with STF-62247. After staining, all protein bands from both gels (SILAC and INV) were processed by LC-MS/MS followed by bioinformatics analyses with IPA (Fig. 1a, right).

Nearly 3,000 proteins were identified in each STF-62247 and INV SILAC replicates (Fig. 1b). From this list, we noted 1,088 and 1,170 common proteins between STF-62247 and INV triplicates, respectively. The number of unique proteins for STF and INV experiments was 333 and 415, respectively (Fig. 1b). To reduce these numbers and eliminate off-target deregulated proteins not directly due to STF-62247 treatment, both lists were compared. Unlike STF-62247 SILACs, INV SILACs were performed after light-isotope containing media was treated with STF-62247. For this reason, only upregulated proteins identified as downregulated in INV-SILACs (or downregulated appearing upregulated in INV), were considered. A list of 755 identified proteins was obtained (Fig. 1b). A heat-map with respective significant quantitative values of all six replicates (STF-62247 and INVERT) was generated with a fold change cut-off of 0.25 and 1.25 with a p values of 0.05 (Fig. 1c). Our SILAC workflow was successful in identifying 755 proteins. Most importantly, differentially expressed proteins were validated by INV experiments, showing reproducibility of the method.

**Validation of proteins identified by SILAC and classification of GO annotations using DAVID**

A cDNA microarray (RCC4 vs. STF-62247-treated RCC4) was compared to the SILAC proteomic data and among 87 hits correlating between the two platforms, cathepsin D and p62 were the most upregulated (Fig. 1c, 2a, and 2b). SILAC experiments were performed in VHL-deficient cells, but protein expression was also assessed in cells that surmount STF-62247 treatment stably expressing VHL (RCC4 VHL). The top upregulated proteins identified by SILAC were TMEM59, Cathepsin D and p62 (Fig. 1c). Accordingly, western blots showed an increase in the uncleaved form of Cathepsin D while its active cleaved form decreased (Fig. 2c). In contrast, no apparent decrease of the active form is seen in RCC4 VHL cells. TMEM59 was also validated with a clear increased expression in response to STF-62247 treatment while RCC4 VHL responded in an opposite manner, with a time-dependent decreased expression (Fig. 2c). No significant change in expression was quantified by SILAC and western blot analysis for UBA1.

We interrogated the DAVID to acquire gene ontology (GO) domains of quantified proteins identified by SILAC (Fig. 2d). Interestingly, many protein biological functions refer to protein localization, protein transport (vesicle-mediated transport, golgi vesicle transport or endosome transport), regulation of protein ubiquitination, protein translation or protein folding, all of which are directly related to dynamic processes such as autophagy and endocytosis. GO_Cellular component results were also closely associated with the above-mentioned biological functions, including membrane-enclosed lumen, organelle membrane, endoplasmic reticulum, vesicle, golgi apparatus and transport vesicle (Fig. 2d). These results showed that expression of top upregulated proteins identified by SILAC proteomics corroborate at the mRNA level and were validated by western blot.

**Deregulated signaling pathways in RCC4 cells predicted by ingenuity pathway analysis**

IPA was utilized to analyze SILAC data and link deregulated proteins to affected signaling pathways. Autophagy was of...
Figure 1. SILAC experimental procedures and data compilation. (a) Experimental procedures of SILAC. (i) For complete incorporation of isotopic-labeled amino acids, RCC4 cells were grown in light and heavy isotope-containing media for five cell doublings. Proteins lysates were pooled in different ratio quantities (heavy/light) and migrated by SDS–PAGE. One band from each lane was excised and trypsin digested for LC-MS/MS analysis. (ii) Cells in heavy media were treated at 1.25 μM STF-62247 for 48 hr. Proteins from light and heavy-treated conditions were mixed (1:1) and migrated by SDS–PAGE. Bands were excised, trypsin digested and processed by LC-MS/MS. Invert SILAC experiments consists in treating light-isotope containing cells at 1.25 μM STF-62247 while cells in the HEAVY condition were used as control. (b) Venn diagrams show quantified proteins in three biological replicates for SILAC STF-62247 and INVERT SILAC. (c) Heat map of significant deregulated proteins of all six replicates.
interest to us as our previous study linked STF-62247 selectivity to this pathway. Interestingly, IPA generated a schematic view of up-(red) or down-(green) regulated proteins having direct roles in autophagy and linked them to predicted affected pathways; endoplasmic reticulum stress pathway, unfolded protein response, p70S6K, AMPK, mTOR and

Figure 2. Validation of proteins identified by SILAC and classification of GO annotations using DAVID. (a) Heat map of cDNA microarray transcriptomic data for RCC4 vs. STF-62247-treated RCC4 cells. (b) Venn diagram comparing SILAC with transcriptomic data. (c) Western blots for Cathepsin D, TMEM59 and UBA1 in RCC4 and RCC4 VHL cells treated at 1.25 μM of STF-62247 for 0 to 48 hr. (d) Histogram of gene ontology annotations (biological function; cell component). GO annotations for 755 deregulated proteins analyzed by the database for annotation, visualization and integrated discovery (DAVID).
Figure 3. Deregulated signaling pathways in VHL-null cells after STF-62247 treatment by ingenuity pathway analysis. (a) Quantified overexpressed (red) and under expressed (green) proteins in response to STF-62247 with direct roles in autophagy. Quantification values are directly proportional to the intensity of the color, i.e., light green/red as less deregulated as dark green/red. Signaling pathways predicted to be affected by the small molecule and having a direct influence on autophagy are illustrated. (b) Histogram of signaling pathways with corresponding –log p(value) and z scores. Positive z scores indicate a prediction of an activation of the signaling pathway whereas a negative z scores indicates an inactivation. (c) Network representing affected interconnected signaling pathways. (d) Schematic representation of mTOR/p70S6K signaling generated with IPA.
Nuclear Factor, Erythroid 2 Like 2 (NRF2)-mediated oxidative stress response (Fig. 3a). These pathways were further confirmed by a histogram generated by IPA based on -log(p values) and represented by their interconnection (Figs. 3b and 3c). The z-scores serve as predictions in activation or inactivation of identified pathways represented in orange and in blue, respectively. Remodeling of epithelial junctions and NRF2-mediated oxidative stress—response were pathways predicted to be activated in response to STF-62247 (Figs. 3b and 3c). A major pathway directly affecting autophagy predicted to be inactivated in response to STF-62247 is mTOR and its downstream effectors, p70S6K, leading to protein synthesis (Fig. 3d). Because mTOR is known to be a major regulator of autophagy, this was of interest to be further validated.11,12 IPA analyses were able to link protein expression quantified by SILAC to predicted affected pathways giving an idea on STF-62247’s effect in ccRCC cells lacking VHL.

NRF2-mediated oxidative stress response is not triggered in response to STF-62247
To validate some predictions by IPA, dysregulated proteins in two above-mentioned pathways; Remodeling of Epithelial Adherens Junctions and NRF2-mediated oxidative stress were briefly explored. Activation z-scores were predicted by IPA for Rab5 and Rab7, two small GTPases.26 Western blots briefly explored. Activation z-scores were predicted by IPA based on -log(p values) and represented by their interconnection (Figs. 3b and 3c). The z-scores serve as predictions in activation or inactivation of identified pathways represented in orange and in blue, respectively. Remodeling of epithelial junctions and NRF2-mediated oxidative stress—response were pathways predicted to be activated in response to STF-62247 (Figs. 3b and 3c). A major pathway directly affecting autophagy predicted to be inactivated in response to STF-62247 is mTOR and its downstream effectors, p70S6K, leading to protein synthesis (Fig. 3d). Because mTOR is known to be a major regulator of autophagy, this was of interest to be further validated.11,12 IPA analyses were able to link protein expression quantified by SILAC to predicted affected pathways giving an idea on STF-62247’s effect in ccRCC cells lacking VHL.

STF-62247 decreases mTORC1 activation differentially from Rapamycin and RAD001
Inactivation of mTOR signaling pathway leading to protein synthesis arrest was also predicted by IPA analysis of SILAC data (Fig. 3d). Thus, mTOR and some of its downstream effectors were investigated to validate IPA predictions and SILAC data. In cells lacking VHL, expression of P-mTOR (S2448) decreased in a time-dependent manner in response to treatment and is further confirmed by a decreased expression of its downstream effectors P-p70S6K (T389) and P-4EBP1 (T37/46) (Fig. 5a). Contrastingly, P-mTOR (S2448) levels in RCC4 VHL cells return to normal states at the 48 hr time point with a concurring expression pattern for P-p70S6K (T389) (Fig. 5a). Based on these results, mTORC1 would seem to be reactivated in cells expressing VHL coinciding with increased levels of P-4EBP1 (T37/46) (Fig. 5a). These results confirm IPA predictions and indicate that STF-62247 acts on mTORC1 phosphorylation and activation.

To compare the effect of STF-62247 with known allosteric mTOR inhibitors (Rapamycin and RAD001), western blot analysis and XTT assays were performed. Both inhibitors decreased mTOR phosphorylation in RCC4 cells with STF-62247 after 48 hr of treatment (Fig. 5b). Phosphorylation of mTOR downstream effector p70S6K (T389) is also decreased in both cell lines at 24 and 48 hr time points confirming mTOR downstream signaling inhibition. XTT assays showed a selectivity of STF-62247 for VHL-deficient RCC4 cells while none is observed when cells are treated with either RAD001 or Rapamycin alone (Fig. 5c). To monitor possible synergistic effects of mTOR inhibitors with STF-62247, increasing concentrations of either Rapamycin or RAD001 were combined with STF-62247 (Fig. 5d). When combined with STF-62247, both inhibitors abolished the specificity of the compound for VHL-deficient cells suggesting a different upstream signaling associated with STF-62247 (Fig. 5d). Unlike allosteric mTOR inhibitors that show a same inhibitory effect regardless of VHL status, these results suggest that STF-62247 sustains a decreased phosphorylation of mTOR and its downstream effectors in a selective manner, dependent on VHL status.

STF-62247 decreases protein synthesis in VHL-deficient cells
To further explore the implication of mTORC1 inhibition, we employed two approaches to measure its downstream effect on protein synthesis. Firstly, to measure global protein synthesis, Click-iT HPG AlexaFluor protein Synthesis Assay showed a decrease in protein synthesis in VHL-deficient cells from 0 to 48 hr as assessed by fluorescence intensity (Fig. 6a). Cycloheximide (CHX), a protein synthesis inhibitor was used as a positive control and abolished Click-iT fluorescence. Concurrent with western blot results obtained for mTORC1 in VHL-proficient cells (Fig. 5), protein synthesis is shown to be unaffected, even slightly increased in RCC4 VHL treated cells with STF-62247 (Fig. 6a). Secondly, we optimized a quantitative protocol using labeled phenylalanine previously developed by Lamarre et al.

The graph presented in Figure 6b shows individual experiments (N = 1,2,3) in both RCC4 and RCC4 VHL from 0 to 48 hr. K_r (% day^{-1}) is representative of protein synthesis levels. These levels decreased significantly in RCC4-treated cells after 24 hr (Fig. 6b). At 48 hr, protein synthesis slightly increased in RCC4 albeit staying significantly lower compared to the untreated control. Again, protein synthesis seems to be increasing at 48 hr in VHL-containing cells in response to STF-62247 supporting the results obtained by the Click-it fluorescence (Fig. 6b) and reflecting mTORC1 phosphorylation states showed previously (Fig. 5a).
Figure 4. NRF2-mediated oxidative stress response is not triggered in response to STF-62247. (a) Western blot analysis of endocytosis-related Rab5 and Rab7 present in this pathway. (b) RCC4 and RCC4 VHL cells treated at 1.25 μM STF-62247 for 24 hr and incubated with 10 μM H2DCFDA. Data are represented relative to control cells. (c) Translocation of NRF2 to the nucleus monitored by immunofluorescence and confocal microscopy with 1.25 μM STF-62247 for 48 hr. (d) Western blot for p62 in RCC4 and RCC4 VHL cells treated at 1.25 μM STF-62247 from 0 to 72 hr.
Figure 5. STF-62247 decreases mTORC1 activation differentially from Rapamycin and RAD001. (a) Western blot of mTOR, P-mTOR (S2448), p70S6K, P-p70S6K (T389) and P-4EBP1 (T37/46) in RCC4 and RCC4-VHL cells treated at 1.25 μM of STF-62247 for 0 to 48 hr. (b) Western blot of mTOR, P-mTOR (S2448), p70S6K and p-p70S6K (T389) in RCC4 and RCC4-VHL cells treated with 100 nM Rapamycin or 10 nM RAD001 (0–48 hr). (c) XTT assays in RCC4 and RCC4 VHL cell lines treated with STF-62247, Rapamycin or RAD001 alone. Combination of 1.25 μM of STF-62247 with a concentration range of 0–80 nM of Rapamycin or 0–3.2 μM of RAD001. Mean and SEM were calculated from four independent experiments. Statistical analysis compared untreated cells with treated cells (*p < 0.05, **p < 0.01, ***p < 0.001) and the difference between the two cell lines (##p < 0.01, ###p < 0.01) at each concentration using Student’s t test.

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Figure 6. STF-62247 induces reduction in protein synthesis in VHL-deficient cells. (a) Protein synthesis measurement by Click-iT HPG Alexa-Fluor protein Synthesis Assay Kit. RCC4 and RCC4-VHL cells treated at 1.25 μM STF-62247 for 0–48 hr or with 100 μg/mL of cycloheximide (CHX) for 1 hr. Protein synthesis was determined by fluorescence on a confocal microscope. (b) Fractional rate of protein synthesis was measured based on S.G. Lamarre et al.’s method and adapted for mammalian cells as described in materials and methods. (c) Western blot for efficiency of EEF2 silencing with siEEF2 and nontargeting siRNAs. EEF2 was detected 72 hr posttransfection. Both membranes (RCC4 and RCC4-VHL) have been immunodetected at the same time. (d) Quantification of band intensities for EEF2 in relation to β-actin for all conditions presented in Figure 6c. Statistical analysis compared the relative levels of EEF2 for each siRNA treated cell lines relation their control using Student’s t test. *p < 0.05. (e) Clonogenic assay of EEF2 silencing compared to the mock transfection (control) and nontargeting (NT) siRNA. Cells were plated 72 hr posttransfection and treated with 0–2.5 μM at STF-62247. Colony formation was quantified after 9 days incubation. Relative quantification was compared to cells transfected with the nontargeting sequence. Statistical analysis compared the relative levels of survival observed in siEEF2 cells to siControl cells using Student’s t test (*p < 0.05, **p < 0.01, ***p < 0.001).
To explore the difference in protein synthesis levels observed between VHL-null and cells with a functional gene, we evaluated the effect of EEF2 inhibition on clonogenic ability. Knock-down of EEF2 was achieved by siRNA and compared to a nontargeting control (Figs. 6c and 6d). As previously reported, survival of RCC4 cells decreased in a concentration dependent manner while survival for RCC4 VHL remained unaffected.\(^1\) Interestingly, knockdown expression of EEF2 in VHL-deficient cells caused a synergistic effect and further decreased survival of RCC4 cells in presence of 0–2.5 \(\mu\text{M}\) STF-62247. In contrast, cell survival was largely unaffected in VHL cells silenced for EEF2 (Fig. 6e). Taken together, SILAC quantification and IPA bioinformatics analyses helped us identify some of STF-62247 mechanisms of action in RCC4 \(~/-\)VHL. We have validated IPA predictions and showed that STF-62247 acts on phosphorylation of mTOR and its downstream effectors and significantly decreases protein synthesis, as measured by two methods.

**Discussion**

To date, available HIF-targeting therapies for the treatment of mRCC remain inefficient and patients too often live a progression of their metastatic disease. The previous identification of a small molecule compound STF-62247 has shown that targeting the loss of VHL could be an attractive new approach to target kidney cancer cells and spare normal tissue. The understanding of its mechanism of action is crucial for the development of novel treatments for mRCC. Our previous studies linked STF-62247 to a deregulation of autophagy and inhibition of key autophagy related genes (ATG) prevented VHL-null cells from death suggesting that this dynamic process was at least in part implicated in the cytotoxicity of this small molecule.

In our study, we used SILAC to paint a global picture of STF-62247 signaling in the cell and most importantly, to identify key pathways that could lead to a better understanding of the molecule’s selective mechanism of action. We employed a global proteomic labeling approach rather than a targeted strategy to quantify and identify all proteins that were modulated in response to STF-62247. We identified 1,088 proteins that were similar between the biological triplicates. The INVERT SILAC was performed to eliminate possible off-target proteins. These steps made possible the identification of 755 proteins in response to STF-62247. Several upregulated proteins were involved in lysosomal processes such as Cathepsin D, p62, lysosomal hydrolases and \(\nu\)-ATPases pump subunits. In addition, we compared proteomics results with transcriptomic profiling performed by microarray in our laboratory. By investigating the 87 differentially expressed proteins that showed correlation at the mRNA level, Cathepsin D, p62 and lysosomal enzymes were present in both analyses. Western blot analysis for Cathepsin D in VHL-deficient cells indicated a clear increase in its pro-form while its active cleaved form was decreased supporting our ongoing findings suggesting that STF-62247 affects lysosomal integrity in these cells.

We used gene ontology to facilitate association of altered proteins quantified by SILAC and their implication in different biological processes, molecular functions and cell components. The functional annotation tool DAVID showed that most proteins had roles in trafficking functions linking autophagy and endocytosis. Both pathways are intimately linked and contribute to lysosomal integrity by trafficking of lysosomal hydrolases and \(\nu\)-ATPase subunits.\(^26,28,29\) Although this tool identified GO annotations associated with SILAC data, it did not consider quantification values and this could explain some differences observed between the pathway analyses by DAVID and those identified by IPA. Nevertheless, bioinformatics analyses using DAVID and IPA lead us to investigate the NRF2 and the mTOR pathway that could be linked to autophagy and lysosomal processes.

By investigating components of predicted pathways, our results showed a decreased phosphorylation of mTOR (S2448) further confirmed by a decrease of direct mTOR effectors, P-p70S6K (T389) and P-4EBP1 (T37/46). Interestingly, inhibition of mTORC1 activation was specific to VHL-null cells, concurrent with protein synthesis arrest, measured by two different techniques. Contrastingly, phosphorylation states of mTOR and of its downstream effectors seem to return to levels comparable in the untreated control in VHL-proficient cells concurrent with stable, even augmented protein synthesis. Moreover, small-interfering RNAs against EEF2 in VHL-deficient cells caused a synergistic effect and further decreased survival of RCC4 cells while remaining largely unaffected in VHL cells silenced for EEF2. Like us, previous studies have also linked VHL status to protein synthesis and have shown that the lack of VHL gene product sensitized RCC cells to the apoptotic effects of a protein synthesis inhibitor.\(^30\) This could be further investigated as a potentially new therapeutic target to bypass development of resistance of RCC tumors to available targeted therapies.

The mechanism by which mTORC1 is inhibited by STF-62247 remains to be elucidated since many upstream sources may contribute to its altered signaling, *i.e.*, amino acids, growth factors and even lipids.\(^16,31–33\) Inhibition of mTORC1 by STF-62247 would seem to be through a mechanism differing from both allosteric inhibitors Rapamycin and RAD001 as they abolish STF-62247 selectivity when combined and do not further decrease cell viability in VHL deficient cells. Lastly, one of the most upregulated protein in response to treatment was the signaling adaptor p62/SQSTM1. We have demonstrated that this over-expression was not due to NRF2-mediated oxidative stress pathway, since no translocation to the nucleus was observed. Recent studies have elucidated central roles of p62 for cell survival and proliferation through activation of mTORC1.\(^34–39\) p62 has been shown to facilitate translocation of mTORC1 to lysosomes but importantly, acts as a scaffold, bringing components involved in
the control of mTORC1 signaling to the correct cellular location. Together, the selective inactivation of mTORC1 in VHL-deficient cells and the deregulated proteins and mRNA associated with lysosomal integrity could lead to hypothesize the possibility that mTORC1 may not be able to get reactivated at the lysosomes, especially if lysosomal membrane components are compromised in response to STF-62247 treatment. This however, remains to be investigated.

Our proteomic study identified signaling pathways implicated in STF-62247 response. Although further mechanistic investigations are needed to shed light on the inhibitory effects of the small molecule on mTORC1, we have validated data on the differences existing in protein synthesis progression and identified a synergistic effect of STF-62247 with knock-down of EEF2. We have succeeded in accomplishing a global proteomic study and have validated bioinformatics analysis and predictions of SILAC data. Although the target of this molecule remains to be identified, this research deepens our understanding of its mechanism of action.

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