Chemical Inhibition of RNA Viruses Reveals REDD1 as Host Defense Factor

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Supplementary Information

Reagents. Human A549, 293T, LnCap, and MDCK cells were obtained from the American Type Culture Collection and cultured in DMEM (Invitrogen) containing 10% fetal bovine serum and 1% penicillin (Invitrogen). Female ICR/CD-1 mouse hepatocytes, InVitroGRO HI and HT Medium, and Celsis Torpedo Antibiotic Mix were purchased from Celsis/In Vitro Technologies (Baltimore, MD). The immortalized human bronchial epithelial cells (HBEC) [1] were obtained originally from John Minna (UT Southwestern) and were cultured in keratinocyte serum-free medium (SFM, Invitrogen). H358 and H1993 cells were also obtained from John Minna. REDD1 cells are immortalized mouse embryo fibroblasts according to a 3T3 protocol [2]. The following antibodies were used for immunoblot analysis: anti-p70 S6 Kinase rabbit polyclonal (Cell Signaling, MA), anti-P-p70 S6 Kinase (T389)(108D2) rabbit monoclonal (Cell Signaling), anti-NXF1/TAP (Proteintech) and anti-P-Akt (S473) (D9E) rabbit monoclonal antibodies (Cell signaling); α-tubulin and γ-tubulin monoclonal antibody (Sigma); goat polyclonal anti-PB1 antibodies (Santa Cruz Biotechnology). phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb (Cell Signaling Technology); REDD1 (DDIT4) rabbit antibody (Novus Biologicals); β-actin (SIGMA); LC3 rabbit antibody (Novus Biologicals). Monoclonal antibody against Complex II subunit 70 kD (Mito-70 kD) was obtained from Mitoscience. Anti-VSV M protein antibody was generated against recombinant full-length protein. Anti-VSV proteins antibodies were a gift from G. Barber. Plasmids pCMVLuc expressing luciferase and pGAGGS-NS1 expressing NS1 were used in the

compound screen and validation assays. The UT Southwestern Compound Library is composed of 200,000 synthetic drug-like compounds purchased from commercial sources and arrayed in DMSO in 384 well plates.

Supplementary Methods

Representative synthetic procedure for the synthesis of naphthalimide analogs

Step 1: Synthesis of 6-(6-chloro-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)hexanoic acid

A solution of 4-chloro-1,8-naphthalic anhyrdie (10.0 g, 43 mmol) and 6-aminocaproic acid (5.6 g, 43 mmol) in *N*-methylpyrrolidone (100 mL) was heated to 110 ºC in a 250 mL round bottom flask under an atmosphere of nitrogen. The reaction was monitored by LC/MS for product formation. After 90 minutes at 110 ºC, the reaction was complete consumption of both starting materials. The reaction was cooled to room temperature and then diluted with ethyl acetate (250 mL) and a dilute aqueous NaCl solution (120 mL). The resulting layers were separated and the aqueous layer back-extracted with EtOAc (2 x 100 mL). The combined organic layers were then washed with water (10 x 100 mL) followed by a final brine wash (50 mL) and the dried over anhydrous Na_2SO_4. Concentration gave a crude dark solid (9.6 g) which was purified using normal phase silica gel chromatography (50% ethyl acetate in hexanes) to provide the desired naphthalimide as a light yellow amorphous solid (7.7 g, 52% yield). ^{1}H NMR ($CDCl_3$) 8.65 (d, 1H), 8.60 (d, 1H), 8.52 (d, 1H) 7.9-7.8 (m, 2H), 4.2 (t, 2H), 2.39 (t, 2H), 1.76 (m, 4H), 1.5 (m, 2H). MS (ESI) 346 (M+H), 368 (M+Na).

Step 2: Synthesis of analog **3**

[6-(1,3-dioxo-6-(piperidin-1-yl)-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)hexanoic acid]

A solution of 6-(6-chloro-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl)hexanoic acid (74 mg, 0.22 mmol, from **Step 1**) in piperidine (2.0 mL) was heated to 80 ^{o}C in a sealed scintillation vial. The reaction was monitored by LC/MS for product formation. After 105 min, the reaction was complete by evidence of complete consumption of the starting material. The reaction was cooled and diluted with EtOAc (20 mL) and then washed with water (~ 10 x 5 mL) until the aqueous washings were clear. The combined aqueous layers were then acidified with 3N HCl to a pH = 4.0 and then extracted with EtOAc (2 x 50mL). The combined organic layers were washed with brine (5 mL) and dried over Na_2SO_4. The crude orange solid obtained after concentration was purified by recrystallization from EtOH to provide **3** as a light orange crystalline solid (40 mg, 46% yield). ^{1}H NMR ($CDCl_3$) 8.58 (d, 1H), 8.49 (d, 1H), 8.38 (d, 1H), 7.53 (t, 1H), 7.18 (d, 1H), 4.20 (t, 2H), 3.21 (br m, 4H), 2.35, (t, 2H), 1.80 (m, 4H), 1.75 (m, 7H), 1.49 (m, 2H). MS (ESI) 395 (M+H), 417 (M+Na).

^{13}C NMR (500 MHz, DMSO) of 3: δ 175.11, 164.17, 163.63, 157.31, 132.88, 131.21, 131.17, 129.76, 126.45, 126.06, 123.12, 115.65, 115.54, 54.63, 34.17, 28.00, 26.73, 26.40, 24.91, 24.54

Purity: 93% at 254 nm

^{13}C NMR (500 MHz, DMSO) of 4: δ 175.12, 164.16, 163.64, 156.09, 149.45, 132.83, 131.32, 131.13, 130.16, 129.72, 128.75, 126.77, 125.98, 123.21, 116.56, 116.42, 115.81, 53.26, 49.56, 34.17, 28.00, 26.73, 24.91, 20.78

Purity: 91% at 254 nm

RNA isolation and real-time RT-PCR

A549 cells were seeded in 35-mm-diameter dishes and infected with A/WSN/1933 virus at an m.o.i. of 0.001 pfu/cell. After one hour, cells were washed and compound-containing medium was overlaid onto the monolayers. At 36 h p.i., the medium was harvested to confirm compound activity by HA assay. From the same wells, total RNA was isolated using TRIzol reagent (Invitrogen) as recommended by the manufacturer. Isolated RNA was treated with 2 U of Turbo DNase (Ambion) at 37°C for 30 min to remove potential genomic DNA contamination. Reverse transcription and real-time RT-PCR was performed by the Mount Sinai Microarray, PCR and Bioinformatics Shared Research Facility as described previously [49]. In brief, cDNA was synthesized using Affiniscript RT (Stratagene) in combination with oligo dT_{18} primers (Integrated DNA Technologies) for cDNA synthesis of cellular genes or an influenza virus NP specific primer for cDNA synthesis of viral NP RNA. Real-time PCR was performed using Platinum Taq polymerase (Invitrogen) and SYBR Green I (Molecular Probes) using an ABI 7900HT real-time PCR machine. Primer nucleotide sequences are available upon request. The results of quantification were normalized to the amount of alpha-tubulin, beta-actin and ribosomal protein S11 mRNA in the same sample. Each PCR was performed in triplicate, and median values and standard deviations were calculated. The amount of RNA was determined with respect to standardized samples and expressed in relative units. A similar assay was performed to measure REDD1 mRNA levels in the absence or presence of actinomycin D (0.5 μg/ml), as described in the legend of Fig. 5a.

Gene Expression Analysis

5 X 10^5 A549 cells, in DMEM media (Gibco) supplemented with 10% FBS (Atlas) and 1% Penicillin (Gibco), were seeded overnight in 6-well plates. Compound **3,** at 30μM, or DMSO

(0.3%) was added for 3 h, and RNA was isolated using the RNeasy Mini Kit (Invitrogen) following the manufacturer's instructions. cDNA was synthesized, labeled, and hybridized to an Illumina HumanRef-8 BeadChip 22K.

After baseline correction and normalization of the expression data from the Illumina bead array, we further filtered the expression profile by omitting entries with a p-value of 0.05 or greater. Fold-changes between test and reference-sets have been calculated. For genes with multiple oligonucleotide probes, fold-changes have been calculated prior to the calculation of averages. Only test and reference pairs for each probe were used when both p-values were at or below the 0.05 cutoff value. We then subjected the post-processed gene expression data to Gene Set Enrichment Analysis (GSEA[50]) using the fold changes as ranks within the *Prerank* algorithm of the GSEA software (http://www.broadinstitute.org/gsea) with default parameters and 2000 permutations. The curated C2/CP "canonical pathways" set from the Molecular Signature Database at the Broad Institute has been used as reference gene sets for GSEA, consisting of sets of genes known to function in 639 pathways. Two enriched gene sets, corresponding to different branches of the same pathway were selected for further response network analysis:

1. MTORPATHWAY: 23 genes (20 genes enriched) on the mammalian target of rapamycin (mTOR) that senses mitogenic factors and nutrients, including ATP, and induces cell proliferation, from BioCarta (http://www.biocarta.com/pathfiles/h_MTORPATHWAY.asp)
2. IGF1MTORPATHWAY: 20 genes (16 genes enriched) on the growth factor IGF-1 that activates AKT, Gsk3-beta, and mTOR to promote muscle hypertrophy, from BioCarta (http://www.biocarta.com/pathfiles/h_IGF1MTORPATHWAY.asp)

For each pathway, the set of enriched genes was used as seed-nodes for further network analysis. NetworkExpress, discussed below, was used to calculate response networks representing the particular response of enriched genes embedded in the constructed human

biochemical network omitting interaction data from iHOP.

Human biochemical network

To construct a hybrid *Homo sapiens* interaction and reaction network, protein-protein interactions with directional signal transduction and metabolic reactions were combined. Interaction information from *IntAct* [3], *NetworKin* [4], the *Human Protein Reference Database* (*HPRD* [5] and from Palsson's group (H. sapiens Recon1@[6] yielded a network of ~40,000 nodes (genes, proteins and small chemicals) as well as ~200,000 interactions (gene-protein, protein-protein) and reactions (chemical, protein-phosphorylation, etc). In addition, curated information on the influenza virus life-cycle and on host-interactions with influenza factors from the *Reactome* database [7] were included. We have also integrated the human biochemical network above with a larger literature based network available from *iHOP* [8] with 45,041 nodes and 438,567 interactions, which are about 2/3s of 650,000 interactions predicted by Stumpf *et al.* [9]. As a third reference network, the Homo sapiens protein interaction network was downloaded from the *BioGRID* database version 2.0.39 [10], which was generated from literature curation of protein interaction data. The data set was filtered to include only direct and physical interactions between human proteins. All loops and duplicate edges were removed. However, duplicate edges from different data sources and different property (e.g., an interaction identified as generic protein-protein interaction in one data-set and predicted as phosphorylation of a protein by a kinase in another data-set) were kept to emphasize the importance/validity of such interactions.

Network Analysis Tool

We have previously developed a computational method to identify response networks in large biological networks based on expression data [11,12]. This method and the corresponding computer program NetworkExpress are based on superimposing expression values upon the

large network, identifying *k*-shortest paths [13,14] between seed-nodes, scoring the sub-network spanned by the set of *k*-shortest paths that are shorter than a pre-defined maximum weighted length *l*, and finding the best scored sub-network by optimization techniques. We have a variety of scoring functions available, from simple arithmetic or geometric means to different types of correlation functions for time-series correlations, optionally between same time-points or time-forward/backward. The best-scored sub-network refers to the response network of the system under the specific environmental condition measured by the corresponding expression experiment. NetworkExpress also performs a statistical analysis to validate the significance of the identified sub-graph by comparison to randomly sampled sub-networks using a Monte Carlo approach.

Protein Synthesis. MDCK cells were seeded into 35mm dishes. Fifty μM of **3** or **8** was added for 6, 24, and 48 hours. Cells were pulsed with 100 μCi ^{35}S-Met for 20 min, harvested, lyzed, and centrifuged at 14,000 x g for 10 min. Supernatant (25μl) was then blotted on Whatman filter paper (approximately 1 square inch) and allowed to dry. The filters were soaked individually in ice cold 10% TCA for 30 minutes. Filters were then washed for 5 minutes with 1:1 ethyl ether/ethanol, and again in ethyl ether alone for 5 minutes. The filters were air-dried and radiation was measured in a scintillation counter.

Supplementary Results

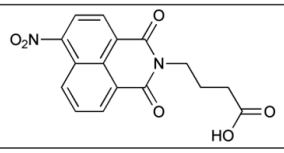
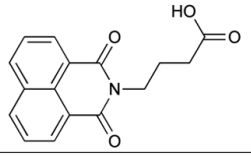
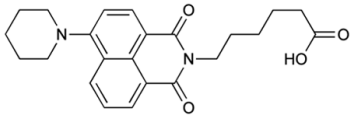
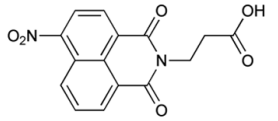
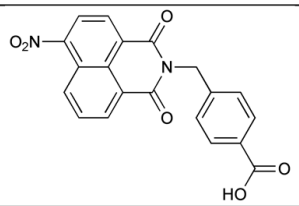
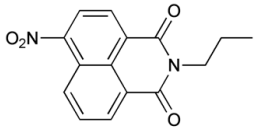
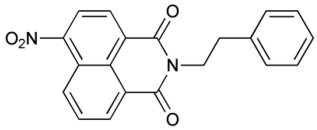
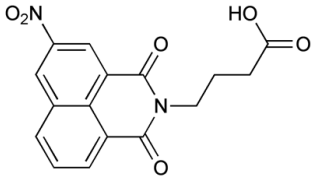
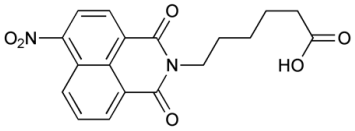
Compound	Structure	Cell Toxicity 24 h	Flu Titer 24 h
1		1.03	0.25
2		1.03	1.0
3		0.88	0.0005
5		0.98	0.1
6		0.62	nd
7		0.36	0.1
8		0.18	nd
9		0.98	nd
10		0.46	nd

Figure S1. Compound 3 is a more potent inhibitor of influenza virus replication than other 1 analogs. To first measure cell toxicity, MDCK cells were treated for 24 h with 20 μM of each compound and ATP levels were measured. Values are normalized to controls treated with DMSO and represent

triplicate values that had standard errors less than 10%. From a separate experiment, supernatants of cells infected with A/WSN/1933 influenza virus (m.o.i. 0.001) and treated for 24 h with 20 μM of each compound were subjected to hemagglutination (HA) assays (data not shown) and plaque assays (shown in the table), and values were normalized to that of control cultures treated with DMSO. Plaque assays were not performed for compounds that exhibited no differences in HA assays (nd). Compound **8** was not tested for virus inhibition due to its rapid cytotoxicity. nd, not determined. Except for **3**, which was synthesized as described above, all the other compounds in this table can be obtained from ChemBridge, ChemDiv, ComGenex, TimTek, and their purities were equal or above 90%.

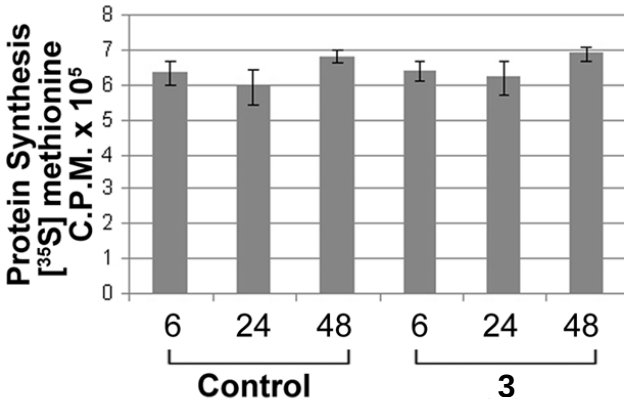


Figure S2. Bulk Protein Synthesis is not inhibited by 3. Protein synthesis was measured by pulse labeling MDCK cells with ^{35}S-methionine, untreated or treated with 50 μM of **3**. Samples were collected at the indicated time points and ^{35}S-methionine labeled proteins were measured.

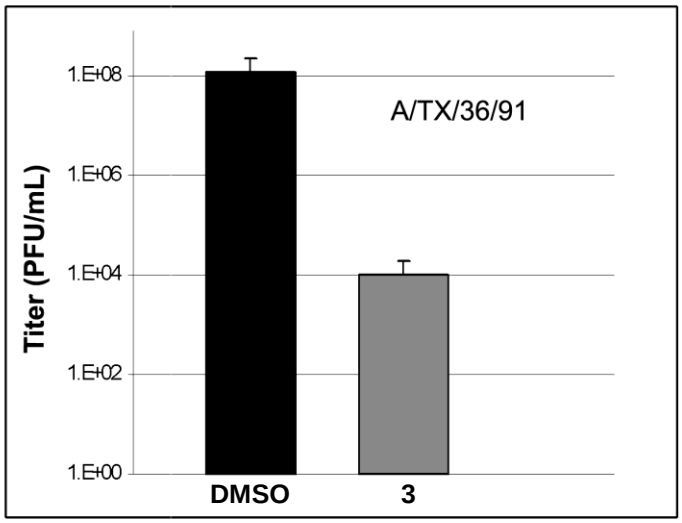


Figure S3. Influenza virus replication is inhibited by 3 in human carcinomic alveolar basal epithelial cells. A549 cells were untreated or pre-treated with 30 μM of **3** overnight. Cells were then infected with A/Texas/36/1991 at m.o.i. 0.001 for 1 h at 22 ^{0}C, in the absence of compound. Cells were shifted to 37 ^{0}C

and **3** was added back to cells that were pre-treated with **3**. Virus titers of culture supernatants harvested at 48 h post-infection were determined by plaque assay.

Supplementary Dataset 1. An additional pdf file is provided with a table showing the microarray data performed in A549 cells untreated or treated with **3** (30μM) for 3 hours in triplicate.

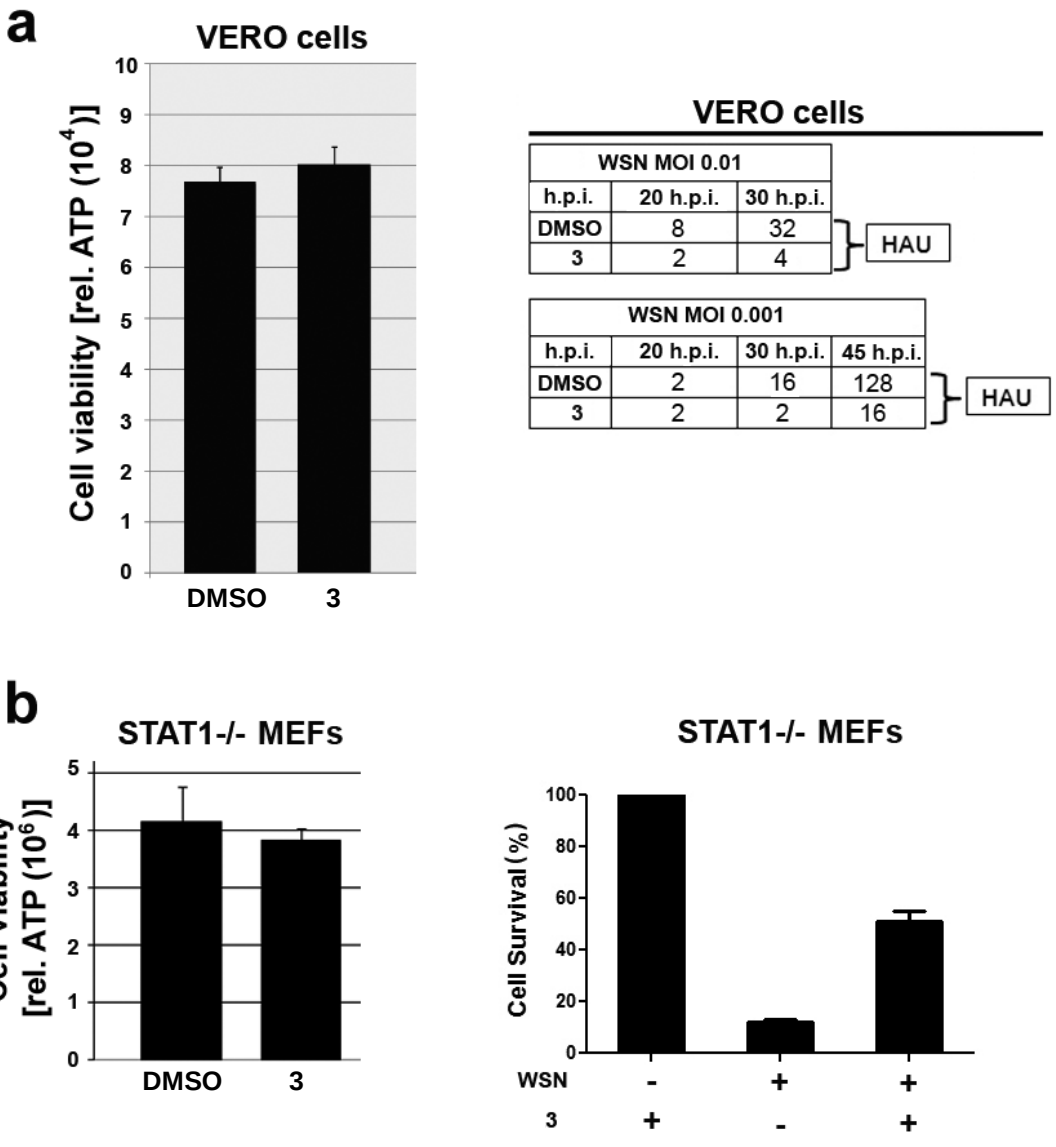


Figure S4. Interferon Response is not Required for Naphthalimide Antiviral Activity. (**a**) Vero cells were untreated or treated with **3** (50μM) and ATP levels were measured. No

cytotoxicity was observed at this concentration. Vero cells were then infected with A/WSN/1933 at an m.o.i. 0.01 or 0.001 for the indicated time points. Cells were treated with DMSO or **3** (50μM) during infection. Supernatants were subjected to hemaglutinin assays (HA) to measure viral titers. HAU, hemagglutination unit. Since Vero cells are interferon-deficient cells and were protected from virus replication by **3**, this compound does not act via interferon. (**b**) STAT1-/- cells were untreated or treated with **3** (40 μM) and ATP levels were measured. No cytotoxicity was observed at this concentration. STAT1 -/- cells were then infected with A/WSN/1933 at an m.o.i. 0.01 for 72 h. Cells were untreated or treated with **3** (40μM) in the absence or presence of virus and cell survival was determined by measuring ATP levels. STAT1-/- cells were significantly protected from viral-mediated cell death in the presence of **3**.

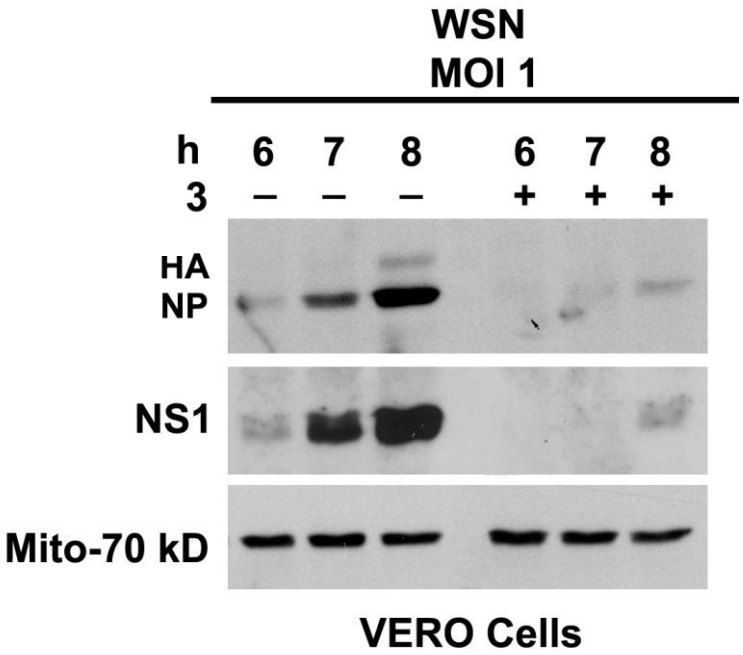


Figure S5. Influenza Virus Protein Levels are Down-Regulated by 3 in Cells with Impaired Interferon Response. Vero cells were pre-treated with **3** (50μM) for 2 h and then infected with A/WSN/1933, at m.o.i. 1, for 1 h in the absence of compound. As shown in Figure S4, **3** is not cytotoxic at the concentration used here. One hour post-infection, **3** was added back and incubated for various time periods as depicted in the figure. Cell extracts were subjected to immunoblot analysis with antibodies against influenza virus proteins or with an antibody against a mitochondrial protein, used as loading control.

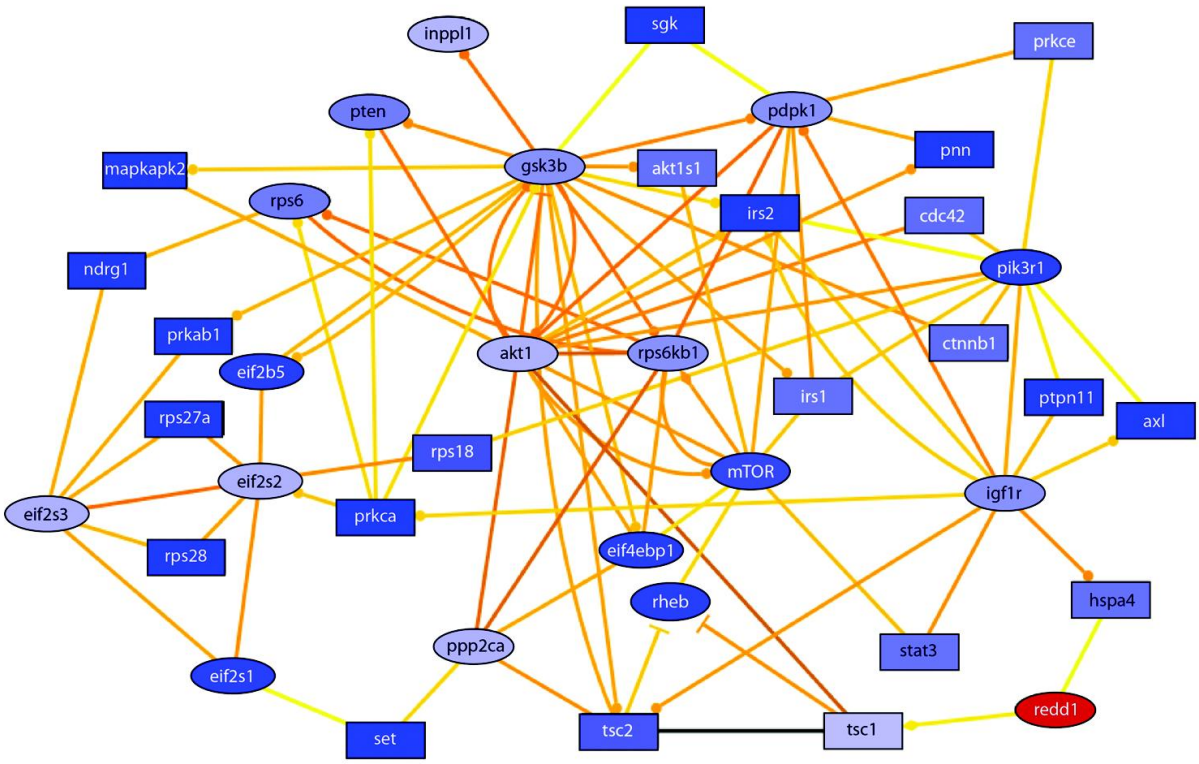


Figure S6. The mTOR Pathway Is Regulated by 3. A549 cells were treated with DMSO or with 30 µM **3** for 3 h. RNA was isolated and processed for microarray analysis. The results of triplicate experiments were subjected to Gene Set Enrichment Analysis as described in Methods. Response networks after enrichment analysis of cells treated with **3** versus DMSO alone are shown: Node colors refer to fold changes - white denoting no change to dark blue indication down-regulation of a three-fold or more, and red depicts up-regulation. Supplementary Dataset 1 shows quantified data. Oval shapes refer to enriched genes in the particular gene set, rectangles denote other genes that have been identified to function in the response network by NetworkExpress. Edge colors indicate edge scores after NetworkExpress analysis using average fold changes between connected nodes. Yellow edges indicate high edge score. Edge arrow shapes denote different types of interactions with arrows indicating metabolic reactions, circles identifying phosphorylation, and no arrow shape refers to protein-protein interactions. The diagram displays the MTORPATHWAY response network with 40 nodes and 92 edges calculated with parameters k= 3 and l= 5.

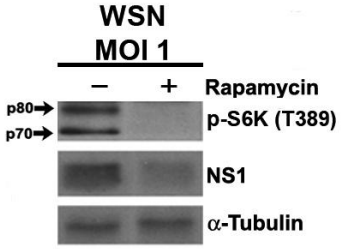


Figure S7. Rapamycin treatment reduced the levels of influenza virus NS1 protein. A549 cells were treated with 100 nM rapamycin for 18 h and then infected with influenza virus A/WSN/1933 for 6 h in the presence of rapamycin. Cell extracts were obtained and immunoblot analysis was performed with anti-NS1 antibodies.

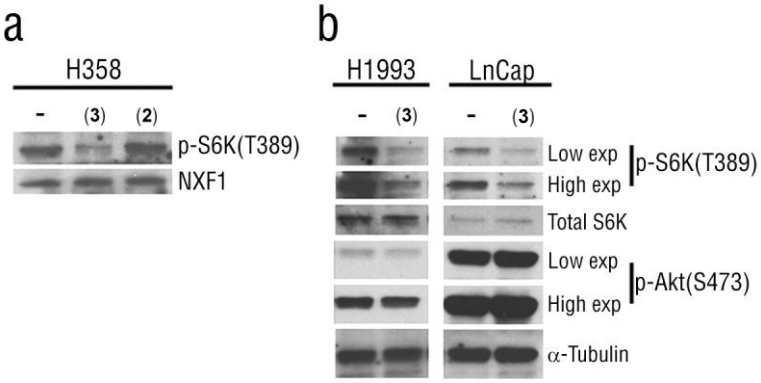


Figure S8. Compound 3 inhibits the mTORC1 pathway independent of virus infection. (**a**) Human lung cancer H358 cells have chronically activated S6K signaling indicated by S6K p-Thr389, which is inhibited by **3** in the absence of virus, but not by **2**. (**b**) In two additional cancer cell lines with chronically activated S6K, **3** inhibited phosphorylation of S6K on Thr389, but did not inhibit Akt phosphorylation.

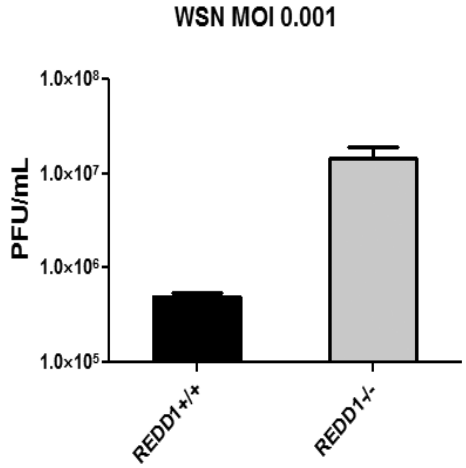


Figure S9. REDD1-/- cells are permissive to influenza virus infection. REDD1+/+ and REDD1-/- cells were infected with A/WSN/1933 at m.o.i. 0.001 for 48 h. Supernatants of infected cells were subjected to plaque assays to determine viral titers.

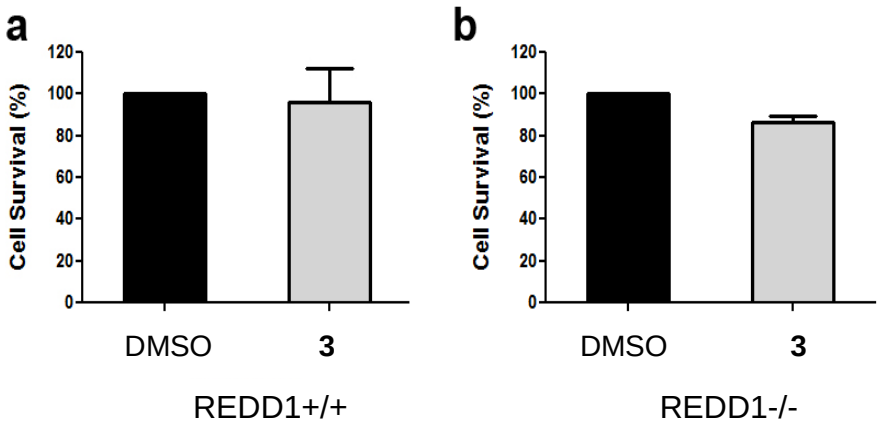


Figure S10. Cell Survival of REDD1+/+ and REDD1-/- Cells Treated with 3. REDD1+/+ cells were treated with 10 μM **3** for 72 h and ATP levels were measured.

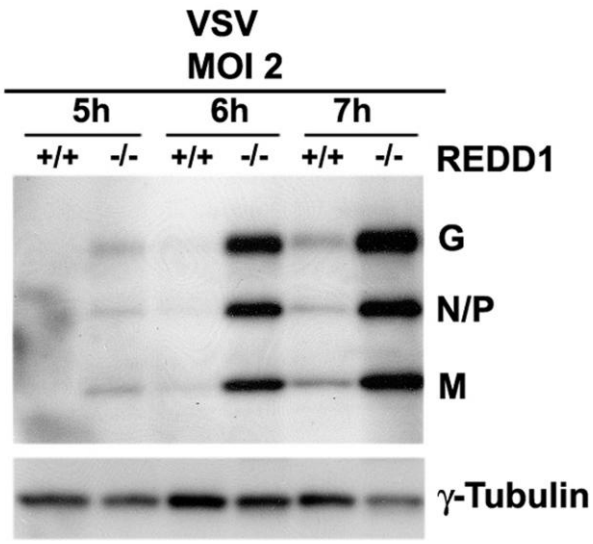


Figure S11. REDD1-/- Cells Express Higher Levels of VSV Proteins than REDD1+/+ Cells. REDD1+/+ and REDD1-/- cells were infected with VSV at m.o.i. 2 for 5h, 6h, or 7h. Cell extracts were subjected to immunoblot analysis with antibodies against VSV proteins.

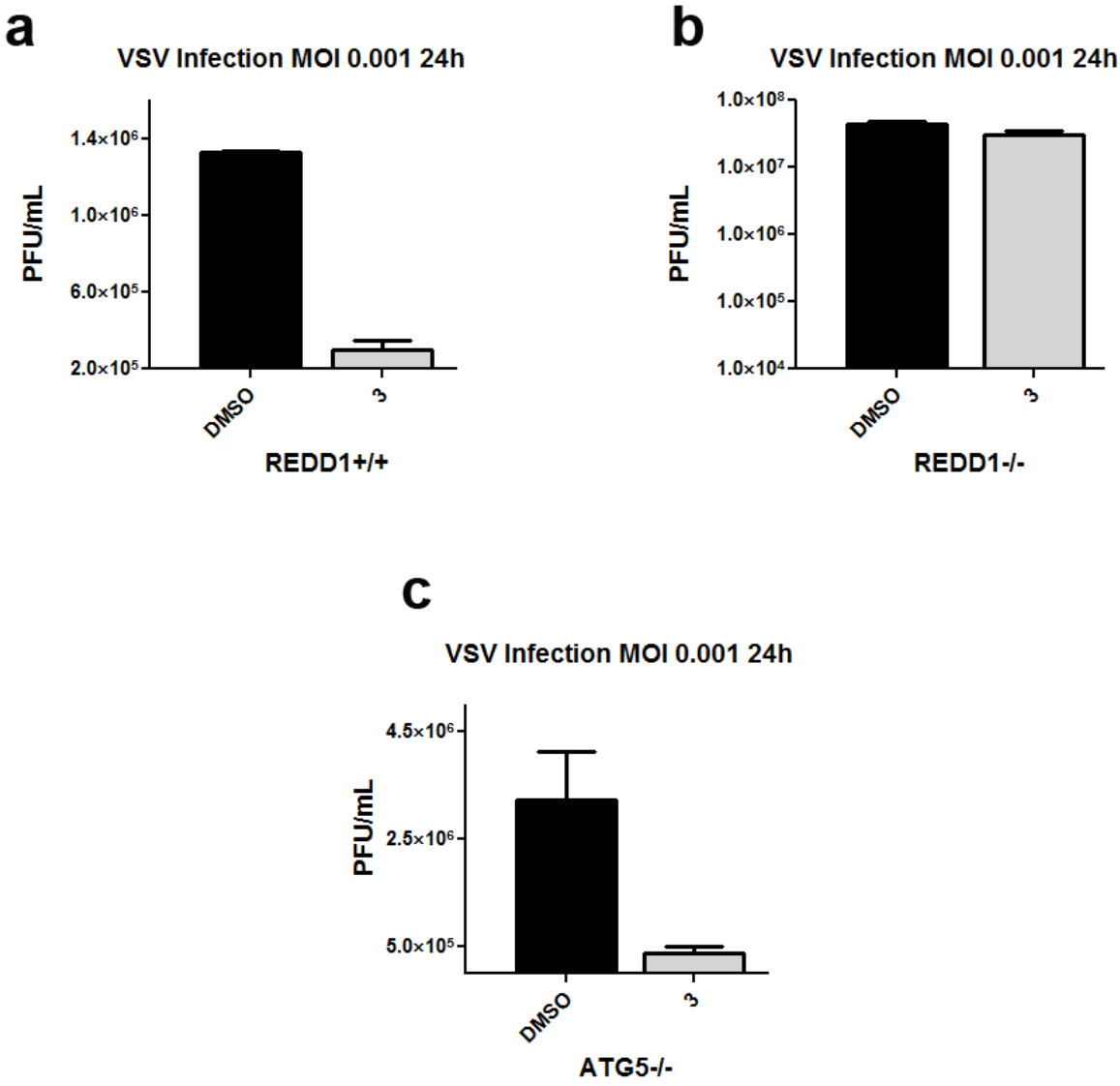


Figure S12. REDD1 is Required for Naphthalimide Antiviral Activity Independent of Autophagy. (**a**) REDD1+/+ or (**b**) REDD1-/- MEFs were untreated or pre-treated for 2 h with **3** (10μM) and then infected with VSV at m.o.i. 0.001 for 1 h in the absence of compound. Then, **3** was added back and infection proceeded for 24 h. Supernatants of infected cells were collected and subjected to plaque assays. (**c**) ATG5-/- cells were infected and processed as in (**a**).

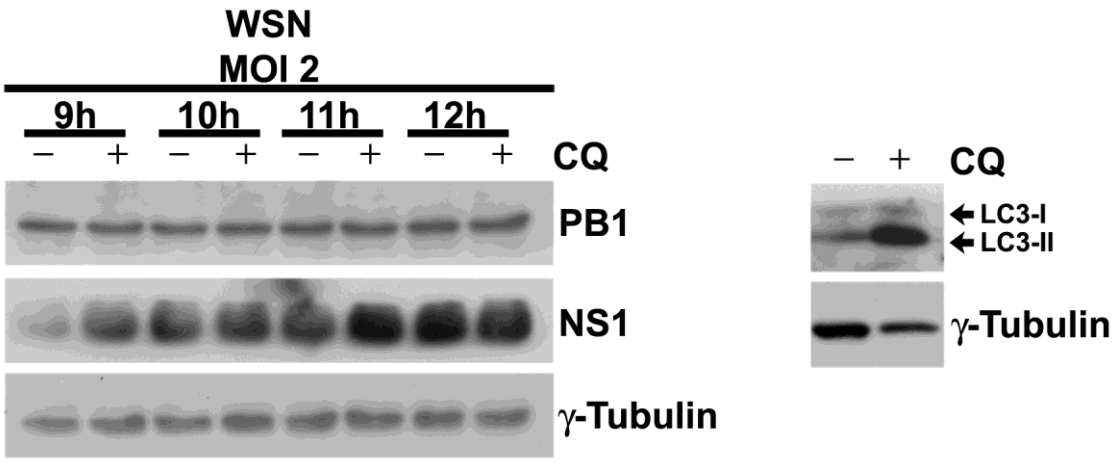


Figure S13. REDD1-/- cells express the same amount of influenza viral proteins in the absence or presence of autophagy inhibitor. REDD1-/- cells were infected with influenza virus over time in the absence or presence of 50 μM chloroquine, which was added 6h post-infection. Cells lysates were subjected to immunoblot analysis with the depicted antibodies. As positive control, LC3-II levels were monitored and were enhanced in the presence of chloroquine treatment. γ-tubulin was used as loading control.

Continue below.

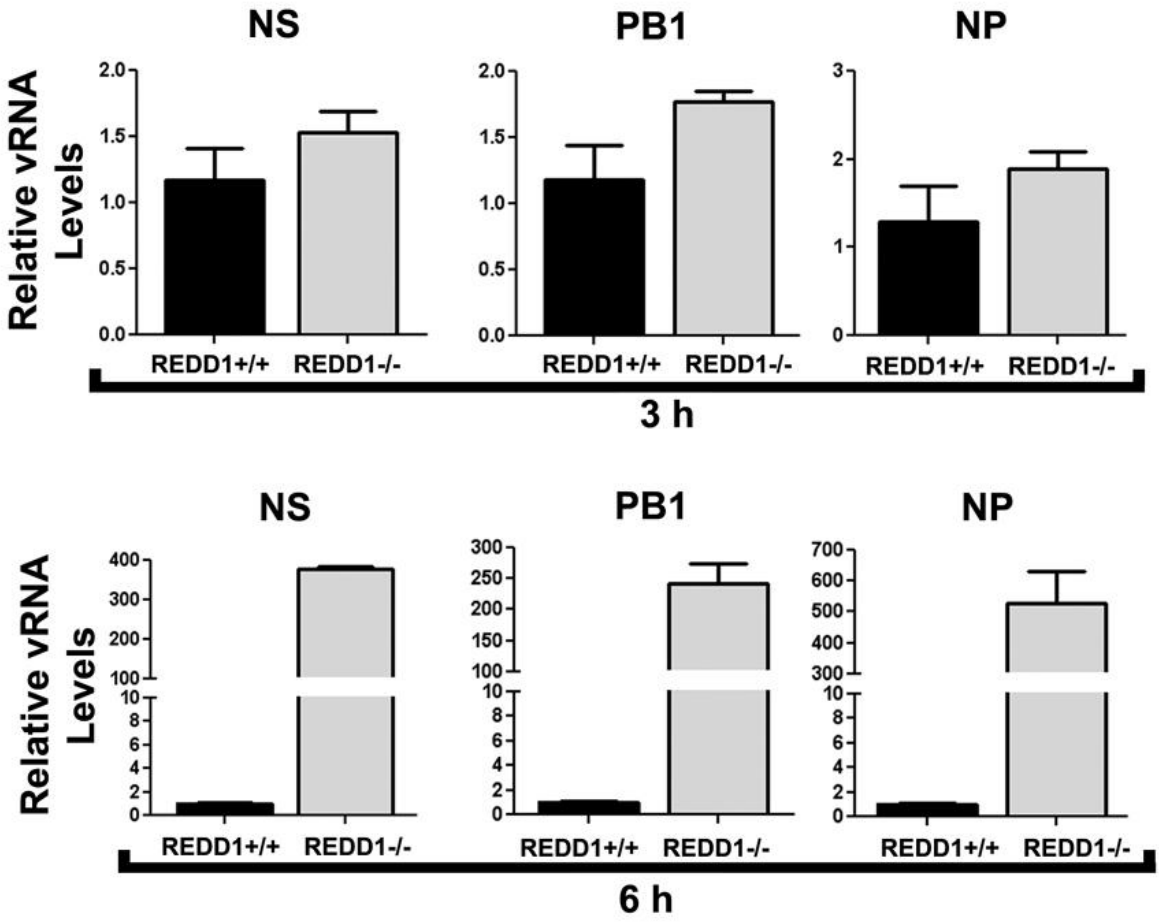


Figure S14. REDD1-/- Cells Express Higher Levels of vRNAs than REDD1+/+ Cells.

REDD1+/+ and REDD1-/- cells were infected with WSN at m.o.i. 2 for 1 h at 22 ^{0}C and then shifted to 37 ^{0}C. Viral RNA (vRNA) levels were monitored by quantitative real time PCR at 3h and 6h. Briefly, total RNA was isolated at 3 and 6 h post-infection using Rneasy Mini Kit (Qiagen, Valencia, CA), following manufacture's protocol. RT was carried out using First Strand cDNA Synthesis using the SuperScript II RT kit (Invitrogen) and specific primers. Real time PCR was performed using gene-specific primers and normalized to HPRT1 and RPS11.

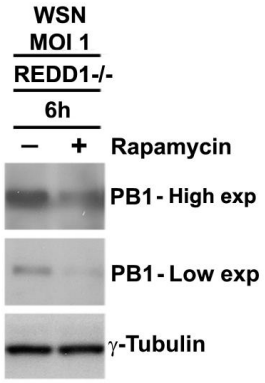


Figure S15. Rapamycin Down-regulates PB1 Levels. WSN-infected REDD1-/- cells were treated with 100nM Rapamycin for 6 h. Rapamycin was added one 1 h post-infection.

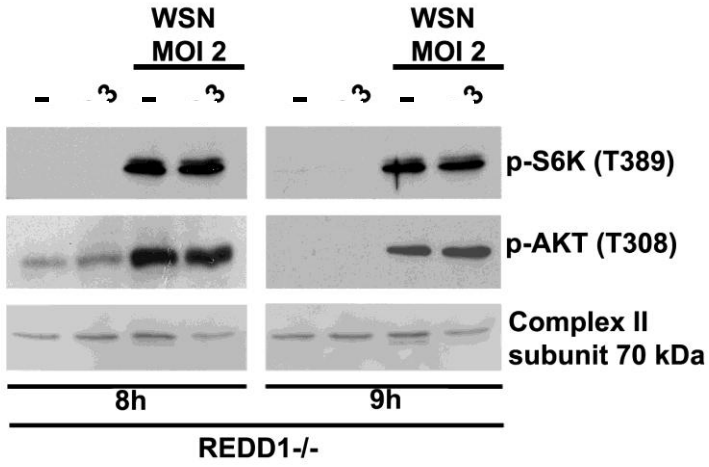


Figure S16. Activation of AKT and S6K is not down-regulated by naphthalimide in WSN-infected REDD1-/- cells. REDD1-/- cells were untreated or treated with **3** and mock infected or infected with influenza virus at m.o.i. 2. Cell extracts were obtained at 8 and 9 h post-infection and subjected to immunoblot analysis with the depicted antibodies.

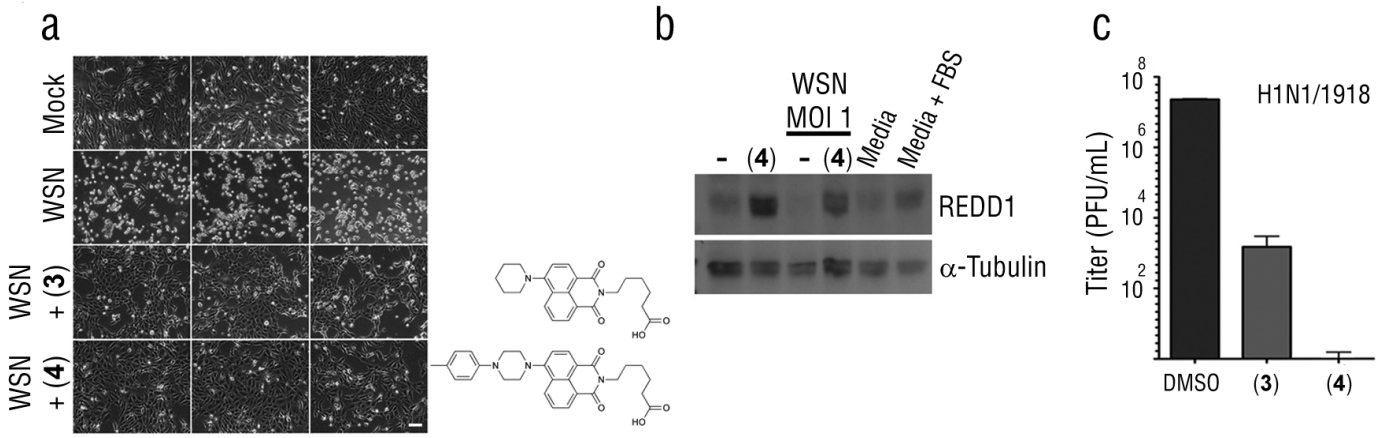


Figure S17. Analog of the naphthalimide 3 more effectively inhibits the highly pathogenic H1N1/1918 influenza virus. (**a**) MDCK cells were pre-treated for 17 h with DMSO or with 25 μM **3** or **4** and subsequently mock infected or infected with A/WSN/1933 at m.o.i. 0.001 for 48 h. Compounds were present during infection. DIC imaging was performed. (**b**) Cells were untreated or treated with 25 μM of **4** and mock infected or infected with A/WSN/1933 at m.o.i.1 for 6h. Cell extracts were subjected to immunoblot analysis with the depicted antibodies. (**c**) H1N1/1918 virus replication in MDCK cells, in the absence or presence of **3** or **4,** was measured by plaque assays.

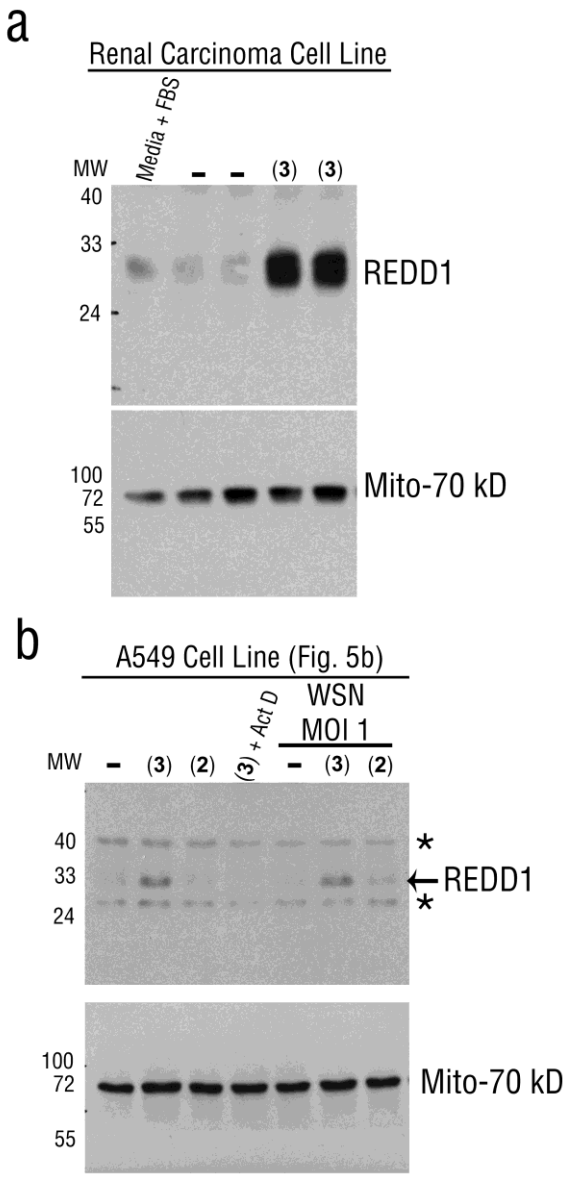


Figure S18. REDD1 is induced by compound 3. (**a**) Renal carcinoma cells were untreated or treated with 20 μM **3** for 6 h. Cell extracts were analyzed by immunoblot analysis with anti-REDD1 or anti-Mito-70kD antibodies. (**b**) A549 cells were untreated or treated with 30 μM **3** (in the absence or presence of 0.5 μg/ml actinomycin D as indicated**)** for 18 h prior to infection and during infection. Cell extracts were obtained at 6 h post-infection and subjected to immunoblot analysis with anti-REDD1 or anti-Mito-70kD antibodies. * cross-reacting bands and Mito-70 kD serve as loading controls. The REDD1 bands are broad in (**a**) because this is a 15-well gel as opposed to (**b**), which is a 10-well gel. In addition, the broadness of REDD1 bands vary between cells types.

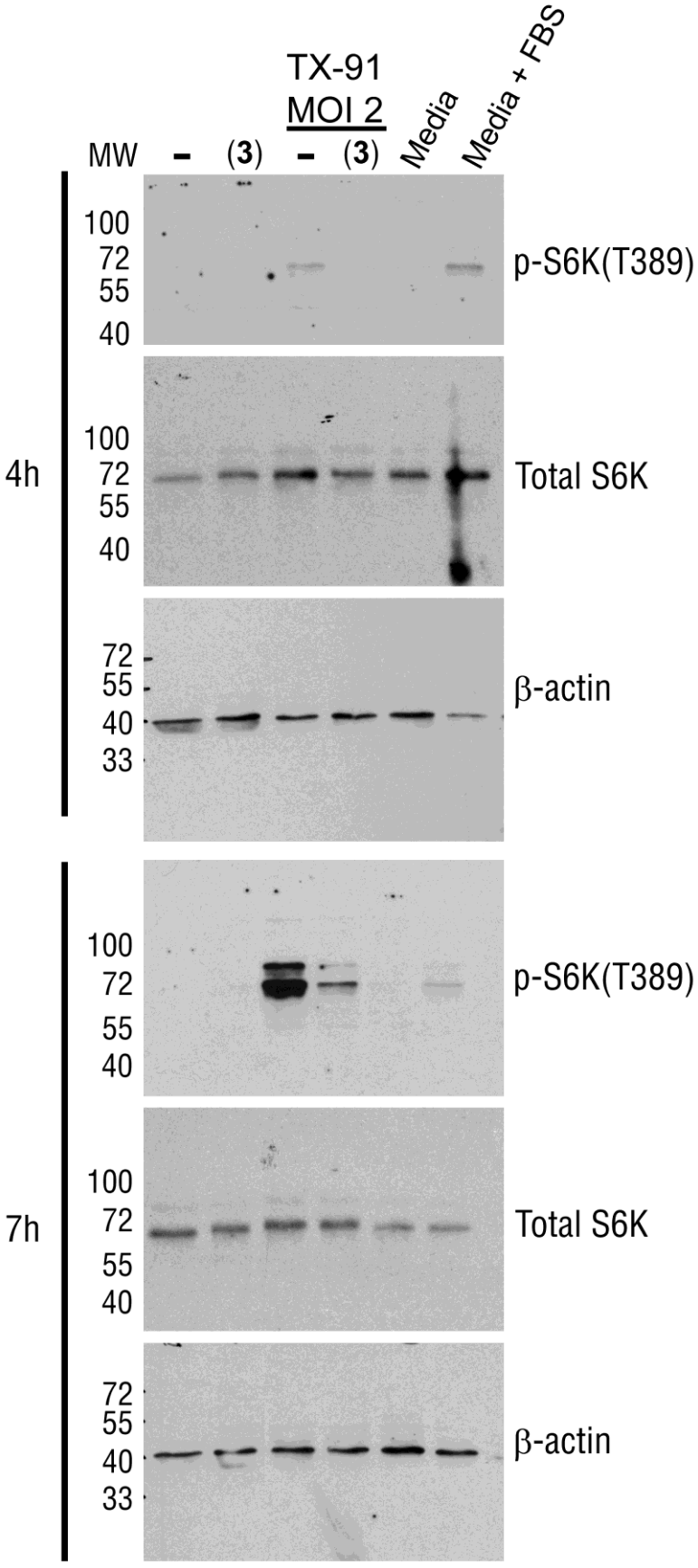


Figure S19. Influenza virus activated the mTORC1 pathway. A549 cells were untreated or treated with 30 μM **3** for 18 h prior to infection and during infection. Cell extracts were obtained at 4 and 7 h post-infection and subjected to immunoblot analysis with the depicted antibodies.

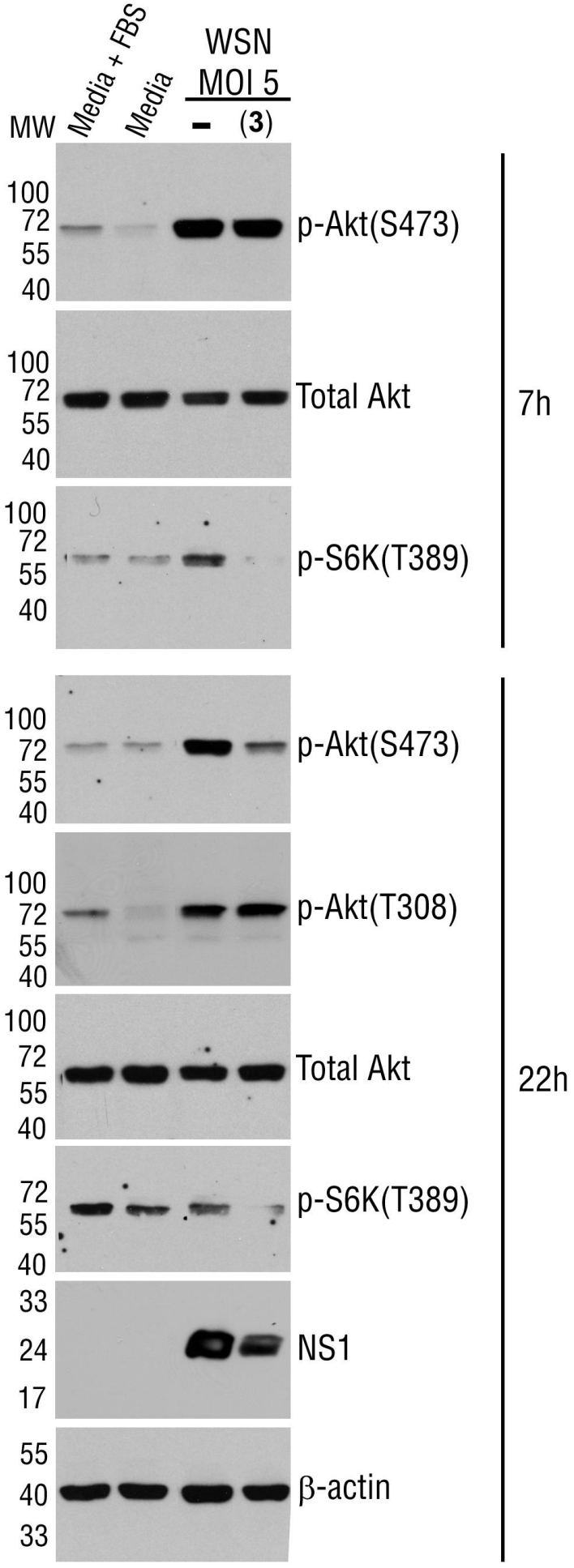


Figure S20. Compound 3 blocks S6K activation independent of Akt. Compound **3** inhibited phosphorylation of S6K in cells infected with influenza virus prior to a decrease in the levels of phosphorylated AKT. Phosphorylation of Akt or S6K was measured by immunoblot analysis in cell extracts of A549 cells infected with influenza virus in the presence or absence of **3**. Compound was added prior and during infection.

Supplementary Table I. Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	Cell-based luciferase reporter
	Target	NS1-mediated inhibition of luciferase expression
	Primary measurement	Luminescence
	Key reagents	pCMV-Luc and pCAGGS-NS1 (ref 18), 293T cells, Lipofectamine 2000 (Invitrogen), Bright Glo (Promega), Eagles Modified Essential Medium + 10% fetal calf serum.
	Assay protocol	293T cells were transfected with an approximately 10:1 ratio of plasmid pCMV-Luc encoding luciferase and pCAGGS-NS1 encoding NS1, using Lipofectamine2000 (Invitrogen). Cells were transfected with the luciferase plasmid alone as a positive control. After 16 hours, cells were dispensed at 5000 cell/well in 384 well plates. After one hour, compounds from the library were added to a final concentration of 5 μM in 1% DMSO in a one compound/one well format. Experimental samples were limited to columns 3 to 22, with controls treated with 1% DMSO in the first and last two columns of wells. Wells in the first column of each plate contained cells transfected with the luciferase plasmid alone; all other wells received cells transfected with both plasmids. Plates were incubated 22 h at 37°C in 5% CO2, then cooled to room temperature and incubated with Bright-Glo luciferase substrate (Promega) for 4 min and luminescence was recorded.
	Additional comments	Care was taken each day to transfect subconfluent cells that had been cultured for 16-18 hours.
Library	Library size	200,000
	Library composition	Commercially available chemical compounds
	Source	ChemDiv, ChemBridge, ComGenex, TimTek
	Additional comments	Compounds in the library represent all the desirable chemical diversity available from the first three companies as of 2004-2005. All compounds passed 48 selective filters and chemical diversity was calculated with CheD software (TimTek).
Screen	Format	384 well plates
	Concentration(s) tested	5 μM
	Plate controls	Column 1 positive control, cells transfected with luciferase alone and treated with final concentration of 1%DMSO. Columns 2, 23 & 24, negative controls transfected with both plasmids and treated with a final concentration of 1% DMSO.
	Reagent/ compound dispensing system	Biomek FX for compounds. Titertek Multidrop for other reagents
	Detection instrument and software	Perkin Elmer Envision and software
	Assay validation/QC	Z' factors were calculated for each plate using the means and standard deviations of the on plate positive and negative controls. Plates with Z' <0.45 were repeated.
	Correction factors	
	Normalization	For each plate experimental values were normalized to the mean of the positive control.
	Additional comments	
Post-HTS analysis	Hit criteria	Hits were defined as wells in which luciferase

	expression exceeded 3.5 standard deviations of the negative controls on the same plate and that had not been previously identified in other screens at UTSW. The top 640 compounds were selected for analysis.
Hit rate	.0032
Additional assay(s)	The primary assay was repeated in triplicate at 15, 5 and 1.67 µM concentrations. All 640 compounds were tested for cytotoxicity on HBEC and MDCK cells, using Celtiter Glo (Promega). All 640 compounds were tested for the ability to inhibit influenza virus cytopathic effects as measured by Celtiter Glo.
Confirmation of hit purity and structure	After resupply from commercial sources or in-house synthesis, purity and identity of compounds was determined by LC/MS with an Agilent 1100 series instrument.
Additional comments	72 compounds were selected as compounds of interest after additional assays were completed. Most of these fell into 8 structure classes and representatives of these 8 were purchased for additional testing.

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