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MECHANISM OF INTERFERON PRODUCTION BY COMPOUND-97

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Jacquelin Crouse

Casey Krawic

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APPROVED:

Robert Finberg, MD
Department of Medicine
UMass Medical School
MAJOR ADVISOR

David Adams, PhD
Biology and Biotechnology
WPI Project Advisor

Abstract

Compounds that stimulate interferon production in cells might have therapeutic effects against viruses that prevent the activation of interferon production. Our lab previously identified compound 97, and its analogs, as potential up-regulators of interferon production, and indicated they might use the MDA5 pathway to achieve this. This project extended this analysis of the mechanism of interferon production using RT-PCR to assay the levels of various mRNAs, bioassays to determine if the induced interferon protects cells against viral infection, nanostring assays to determine the number of mRNA transcripts of 50 interferon inducible genes, and residual viral levels *in vitro* to determine the ability of the compounds to protect against a viral challenge. The results show that the protein RIG-I is necessary for interferon production by compound 97, and the compound can stimulate interferon production in a variety of cell lines (HEK293 cells, macrophages, dendritic cells, and fibroblast cells). Bioassay tests indicated that this compound had the ability to induce bioactivity of the type I interferons (IFN) and protect these cells against the VSV-GFP virus.

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Acknowledgments

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Background

The Immune System

The body is a vast and complicated machine that requires regular maintenance and protection from many harmful organisms. Some organisms are symbiotic with the body, but others are parasitic, using the body for its own means of survival, leading to severe illness and even death. Multicellular organisms are constantly exposed to harmful organisms that can enter the body through air, food, water, and wounds. The body has two phases of immunity, innate immunity and adaptive immunity, depending on the organism and the type of infection (Murphy et al., 2008).

As the first line of defense, the body first enacts the innate immune system. This is a non-adaptive type of defense, and does not usually adapt to repeated exposure to a pathogen, however it can identify a broad spectrum of pathogens. Two of the main cells used by the innate immune system to fight infection are phagocytes, which engulf the invading cell and destroy it using specialized proteins, and natural killer (NK) cells, which recognize and bind to non-self cells via their surface receptors and release cytotoxic granules onto the surface of the bound pathogen. NK cells also secrete effector proteins which puncture the pathogen cell membrane which allows the toxic granules to enter the pathogen to induce apoptosis.

One class of phagocytes includes macrophages. These cells secrete cytokines (such as type I interferon) and chemokines (along with a few other minor proteins) that indirectly act as a calling beacon to other cells that will aid in fighting the infection. Macrophages are derived from monocytes. Monocytes are also the hematopoietic precursors to dendritic cells, and they circulate in the bloodstream to fight infection. Dendritic cells are the major producer of type I interferon. There are two types of dendritic cells, conventional dendritic cells (cDCs) and

plasmacytoid dendritic cells (pDCs), both of which are induced by cytokines. By gathering other cells via the bloodstream, the infection is prevented from gaining access to the body through the blood circulation. Cytokines also cause a reaction called inflammation (Murphy et al., 2008).

Type I Interferons

A specific class of cytokine, and the focus of this MQP project, is the interferon (IFN). There are two main subtypes: α and β . Interferons are produced and secreted along with other cytokines and chemokines after a cell detects an invading virus. These signaling molecules are spread to surrounding cells and are detected by various transmembrane receptors. Interferon α and β are recognized by the interferon α/β receptor (IFNAR) (Figure-1, diagram upper right).

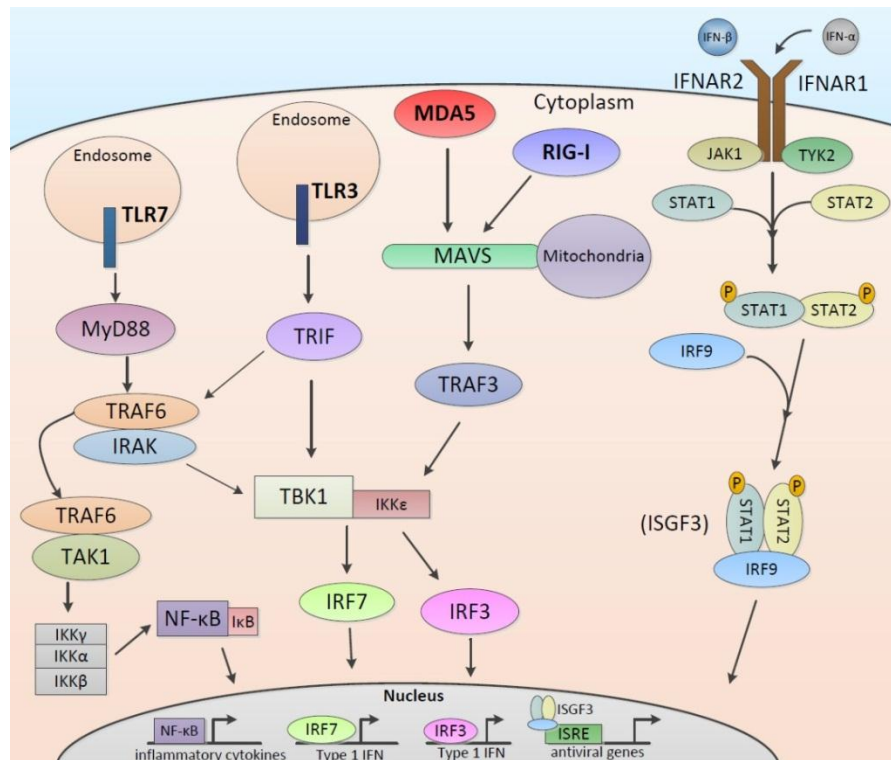


Figure-1: Diagram of Type I Interferon Production and Signaling Pathways.

The binding of type I interferon to the receptor starts the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (diagram right side). JAK1 interacts with IFNAR2, and TYK2 interacts with IFNAR1. When activated, these two kinases phosphorylate STAT1 and STAT2 causing them to dimerize. The dimerized STAT proteins then interact with interferon regulatory factor 7 (IRF7) to create a complex called interferon-stimulated gene factor 3 (ISGF3). This complex is able to enter the nucleus and interact with the IFN-stimulated response element (ISRE). This interaction turns on antiviral genes to fight off the viral infection (Aaronson and Horvath, 2002).

Type I interferons (IFN- α and IFN- β) act to create antigen-presenting cells (APCs) out of macrophages and dendritic cells. Macrophages and dendritic cells contain Toll-Like Receptors (TLR) (diagram left side), of which there are several, that can recognize pathogens and produce the cytokines that call other cells to the infection site. Stimulation of TLRs can activate NF- κ B (diagram lower left), causing the production of cytokines, chemokines, and co-stimulatory molecules. IFN- α and IFN- β cause macrophages and dendritic cells to become APCs when the TLR recognize and bind to the pathogen, then the activated macrophages and dendritic cells act as a recruiting beacon for other macrophages and dendritic cells.

Interferons can also cause the activation of the adaptive immune response by increasing the expression of the Major Histocompatibility Complex (MHC) Class I present on the surface of all host cells. MHC-I molecules bind viral antigens and are recognized by cytotoxic T lymphocytes which kill the infected cell. Plasmacytoid dendritic cells (pDCs) appear to specialize in producing IFN- α and IFN- β , and can secrete up to 1000 times as much interferon as any other cell from the peripheral lymphoid tissues (Murphy et al., 2008).

If the pathogen survives the initial assault, roughly about 4 hours after infection, the adaptive immune system activates, which is able to fight more specifically against a certain pathogen because it can recognize the pathogen using the receptor from the host germline cells to distinguish between self and foreign cells. This second line of defense is able to adapt to various pathogens as they evolve to try to evade the immune system (Murphy et al., 2008).

Type I Interferon Pathways

The production of type 1 interferon is initiated through the activation of two types of receptors: toll-like receptors (TLR) or cytosolic receptors. TLRs are located on the surface of specific cells, or within their endosomal compartments, and recognize nucleic acids from invading viruses. TLR3 is a receptor that recognizes double-stranded RNA. TLR7 and TLR8 detect single-stranded RNA. TLR9 can detect un-methylated CpG motifs in DNA which are common in DNA viruses. With these TLR combined, the immune system can recognize a majority of invading viruses and bacteria (Stetson and Medzhitov, 2006).

All TLRs, except for TLR3, are associated with protein MyD88 (diagram upper left). In the MyD88-dependent pathway, MyD88 recruits TRAF6 and IRAK. This is shown on the left side of Figure-1 starting with TLR7. The pathways continues with TNF-receptor-associated factor 6 (TRAF6) interacting with TAK1 to activate it. Activated TAK1 then activates NFκB through the well-characterized inhibitor-kappa-B (IκB) kinase (IKK) complex. Activated IKK phosphorylates IκB, which becomes released from NFκB, activating it. Activated NFκB stimulates the transcription of pro-inflammatory cytokine genes such as TNF-α, IL-6, and IL-1β, while also activating IFN-β production (Uematsu and Akira, 2007).

The other important adapter molecule of the TLR signaling pathways is TRIF (purple circle, diagram center). TRIF associates with TLR3, and that complex interacts with TBK1 and IKK ϵ . This complex then mediates the phosphorylation of IRF3 and IRF7. Phosphorylated IRF3 and IRF7 are translocated into the nucleus to induce the expression of IFN- α and IFN- β . TLR4 can also initiate the TRIF-dependent pathway, but it requires the adaptive molecule TRAM to bring TLR4 and TRIF together. These pathways are not very linear as seen in the diagram. TRIF can activate TRAF6 and IRAK can activate the TBK1 complex. This causes the activation of one TLR to induce many interferon and inflammatory factors in a variety of ways (Uematsu and Akira, 2007).

Another type 1 interferon activation pathway involves cytosolic receptors. RNA helicases retinoic acid-inducible gene-I (RIG-I) (blue circle, diagram upper center) and melanoma differentiation antigen 5 (MDA5) (red circle, diagram upper center) are able to detect double-stranded RNA to activate the production of type 1 interferon. Both of these receptors use the caspase recruitment domain (CARD) in adaptor protein MAVS to activate the IFN- β gene (Stetson and Medzhitov, 2006). After RIG-I and MDA5 interact with MAVS, the complex interacts with TRAF3 (Takeuchi and Akira, 2008). TRAF3 recruits other complexes and kinases, including IKK ϵ /TBK1, which leads to the phosphorylation of IRF3 and IRF7. IRF3 and IRF7 then act to produce type 1 interferons in the same manner as in the TLR pathway (Garcia-Sastre and Biron, 2006).

History of Interferon Inducer Compounds

In order to treat patients with viral infections, scientists have been investigating antiviral compounds. The first compounds identified were large, and were toxic to the recipient (Hoffman

et al., 1973). During the 1970s, smaller molecular weight compounds were discovered to help mice survive a viral infection and produce interferon with fewer toxic effects. Some compounds were tricyclic in structure, such as tilorone hydrochloride (Krueger and Mayer, 1970), BL-20803 (Siminoff et al., 1973), quinacrine, and acranil (Gláz et al., 1973). Substituted pyrimidines were also tested and showed interferon induction (Nichol et al., 1976; Wierenga et al., 1980). All of these compounds stimulated interferon induction in mice and other mammals, in a dose dependent manner. Some studies also used virus-infected mice to assay potential survival increase after compound administration. The viruses tested included vaccinia virus (Gláz et al., 1973), encephalomyocarditis (EMC) virus (Nichol et al., 1976), semliki forest virus (SFV), herpes simplex virus type 1 (HSV-1) (Wierenga et al., 1980), and various other viruses (Krueger and Mayer, 1970). By performing these survival tests, the amount of compound needed to allow animal survival was determined. By 2001, more information about interferons was known, so new tests could be performed. Two compounds were found: imiquimod and resiquimod (Hirota et al., 2002) which increased the production of IFN- α , and when used in a cream were found to treat the symptoms of human papillomavirus (HPV), HSV-2, or hepatitis-C virus (HCV) (Dockrell and Kinghorn, 2001).

Project Background

As described above, interferons play an important role in the immune response against pathogens, and the ability to induce them can aid in fighting diseases, especially those that the body has difficulty fighting. One such difficult disease is Lassa Fever caused by the Lassa Fever Virus (LFV). LFV is a member of the *Aternaviridae* family, and it infects humans after contact with contaminated rodent blood, feces, or urine, or from other infected humans. The symptoms

can be extreme, ranging from headache, fever, and weakness, to diarrhea, vomiting, hemorrhage and later multi-system failure, leading to death. One-third of patients who survive after the immune system retreat at day 15 are deaf. No Lassa fever vaccine is available, and the only treatment is ribavirin, which is only useful when used within the first 7 days (Baize et al., 2004a).

Lassa Fever is not a rare disease, with approximately 300,000 infections and 5,000 deaths annually, especially in Sierra Leone, Liberia, and Guinea (Kahn et al., 2008). LFV targets macrophages and dendritic cells, which as discussed above, are important for interferon production and fighting infection. However, the actual cause of Lassa Fever has yet to be determined (Baize et al., 2004a). It has been hypothesized that the disease begins with immunosuppression from a lack of activation of dendritic cells (Mahanty et al., 2003), and the virus was also shown to not induce apoptosis in macrophages and dendritic cells. Baize et al. (2004b) also showed that LFV infection did not activate these cells, supporting the Mahanty finding, and there were no observable changes to their surface molecules. There was also no cytokine production at either mRNA or protein levels following infection, leaving the site open to continued infection and allowing the virus access to the blood stream (Baize et al., 2004b).

UMMS Finberg Lab

In this MQP project, Lymphocytic Choriomeningitis Virus (LCMV) is used in place of LFV, as they are members of the same virus family. These viruses are similar in their genomic structure, encoded proteins, and receptors used to infect host cells. The Finberg lab at UMMS is interested in designing potential therapeutics against human viral pathogens that can help the host up-regulate interferon production against these viruses which might block the host type 1

IFN responses. The Finberg lab has shown that LCMV infection causes the up-regulation of several chemokine genes (Zhou et al., 2008). Dr. Shenghua Zhou in the laboratory of Dr. Robert W. Finberg has recently identified a new compound 97, and two of its analogs, 768 and 795, can be used against LCMV infection in human cells, and hypothesizes that this defense might work against LFV infection as well. They further hypothesized that if this new compound can defend against LFV infection, then it may be able to aid against other viral pathogens as well.

Human monocytes were used as the model system used in the preliminary studies. Monocytes are hematopoietic precursors for macrophages and dendritic cells, the major producers of interferon. The Finberg lab identified compound 97 (and its analogs 768 and 795) in a screen of small molecule compound libraries with human embryonic kidney cells engineered to express luciferase reporter gene fused with interferon- β promoter. The cell line was named IFN-b-luc or SZ34. After treating SZ34 cells with various doses of compounds 97, p21, F19 and 6387, the luciferase assay indicated that compounds 97 and p21 caused a large increase in interferon production in a dose-dependent manner (**Figure-2**).

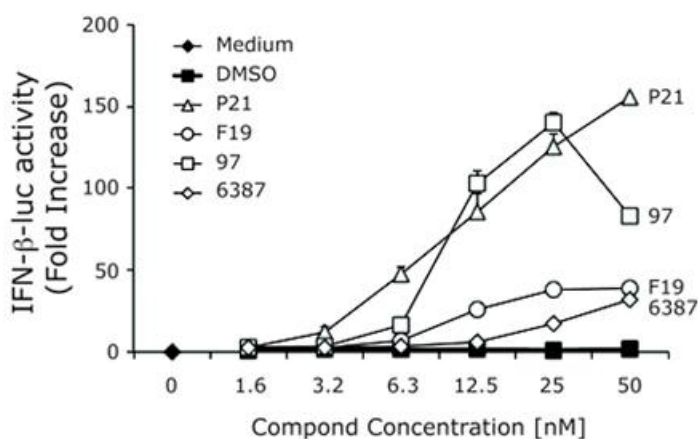


Figure-2: Activation of IFN- β Production from Monocyte Cells by Various Compounds. Human monocyte cells containing a luciferase reporter gene under control of an IFN- β promoter were used to screen a variety of compounds for interferon production at 24 hrs. Y-axis is fold increase of luciferase activity relative to medium alone. (Zhou, 2011)

After observing the increase in β -luciferase, the Finberg lab investigated the compounds' effects on the levels of mRNA by RT-PCR. Human fibroblasts were treated with a compound or control and then assayed after 18 hours for mRNA levels of IFN- β , IFN α 4, IFN-non α 4, MDA5 and RIG-I. GAPDH was used as a loading control. These results showed that compound 97 was a strong inducer of IFN- β and MDA5 in mouse fibroblast cells while p21 was not (**Figure-3**).

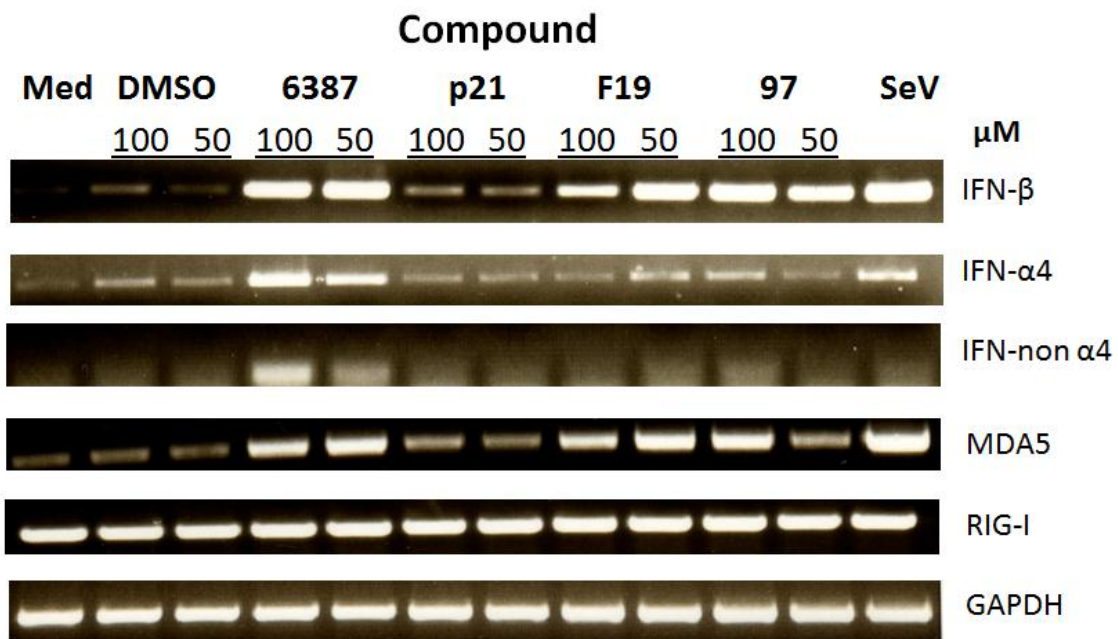


Figure-3: RT-PCR of Various mRNA Levels in Cells Treated with Different Doses of Suspected Interferon Producing Compounds. Human fibroblast cells were treated with various interferon producing compounds for 18 hr, then the mRNA levels were assayed by RT-PCR. Sendai virus (SeV) was used as a positive control for interferon production. (Zhou, 2011)

The data showed that compound 97 was able to induce higher interferon production in human fibroblast cells than p21, so compound 97 was chosen for further testing. Compound 97 was able to induce significant levels of interferon in both mouse and human cells. This ability to induce interferon in both types of cells made compound 97 ideal to study in mouse models before moving on to testing in human cells. Using primary human fibroblast cells, instead of the

SZ34 reporter cell line, Dr. Finberg's lab compared compound 97 and its derivatives for interferon production using a bioassay at 20 hr post-treatment (**Figure-4**). The data indicated that compound 97 and its derivatives increased interferon- β production in fibroblast cells.

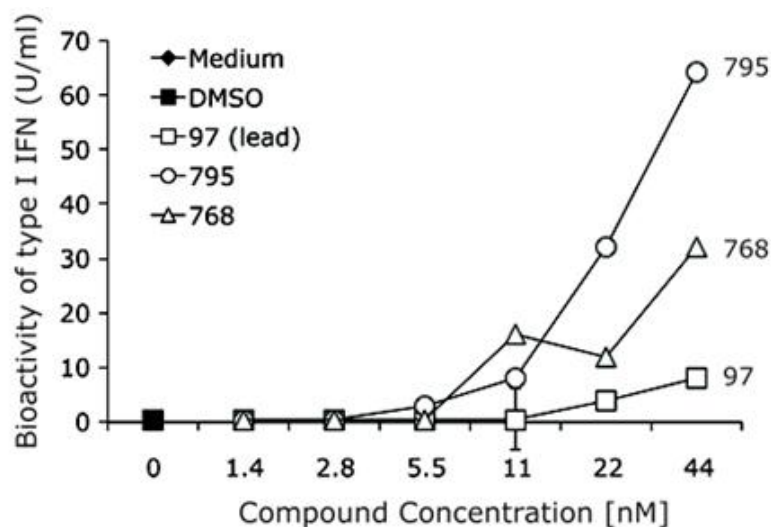


Figure-4: Activation of IFN- β Production from Human Fibroblast Cells by Compound 97 and its Analogs. Interferon production was assayed by a bioassay at 20 hr post-treatment. (Zhou, 2011)

Next, the Finberg lab tested whether the ability of compound 97 and its analogs to increase interferon production could protect human cells from viral infection. Two viruses were used, LCMV and VSV (vesicular stomatitis virus) which are very sensitive to interferons (D'Agostino et al., 2009). Human fibroblast cells were treated with VSV or LCMV for 20 hr, and then the cells were treated with compound-97 or its analogs for 1 hr. Residual virus was measured by GFP for VSV or by immunofocus for LCMV. As seen in **Figure-5**, compound-97 and its analogs lowered viral titers compared to untreated cells.

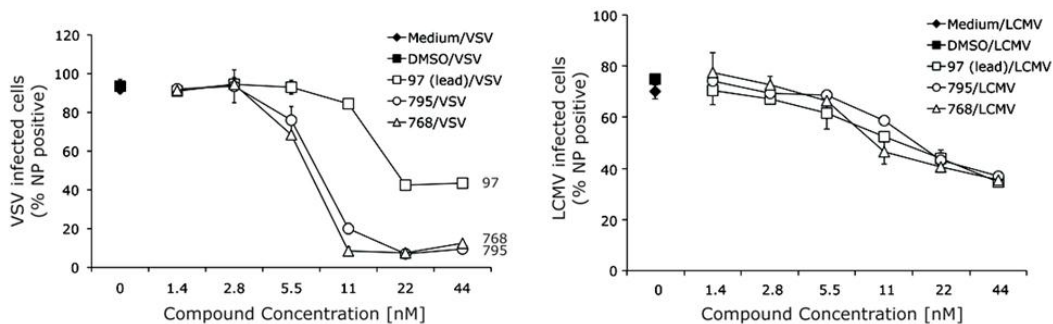


Figure-5: Compound-97 and its Analogs Protect Human Cells from VSV and LCMV Viral Infection. Human fibroblast cells were treated with various interferon producing compounds for 20 hr, infected with VSV or LCMV virus (left and right panels respectively) for 1 hr, then virus levels were measured by GFP expression (left panel) or by immunofocus assay (right panel). (Zhou, 2011)

Based on this data, compound-97 and its analogs were hypothesized to interact with one of the components of the interferon production process. The cellular levels of the mRNA for MDA5, known to participate in interferon production, were assayed by RT-PCR (**Figure-6**). The levels were elevated by the compounds, thus it was hypothesized that MDA5 (and RIG-I which is also associated with the MDA5 pathway) are necessary in the interferon production process by these compounds.

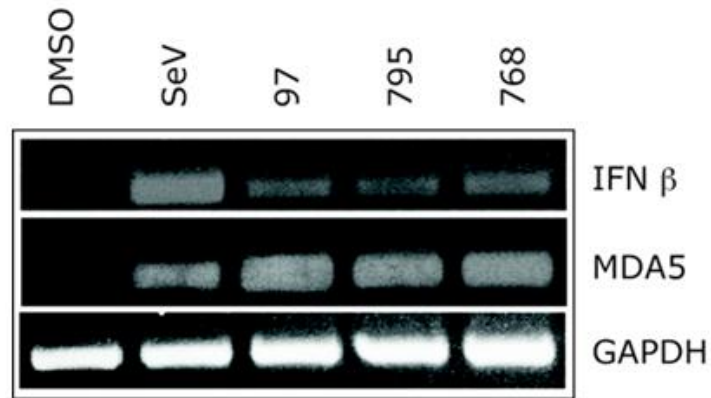


Figure-6: Increase in mRNA Levels of MDA5 by Compound-97 and its Analogs. Human fibroblast cells were treated with various interferon producing compounds for 18 hr, then the mRNA levels for MDA5 were assayed by RT-PCR. Sendai virus (SeV) was used as a positive control for interferon production. (Zhou, 2011)

By hypothesizing that MDA5 and RIG-I are necessary for the production of interferon by compounds 97 and its analogs, a model was designed in **Figure-7** on how interferon is being produced in the cells.

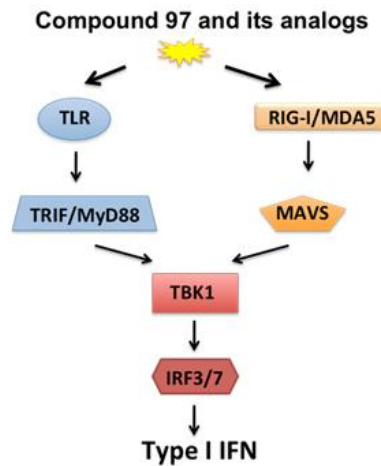


Figure-7: Diagram of a Likely Model for How Compound 97 and Its Analogs Increase Interferon Production in Human Fibroblast Cells.

Project Purpose

Compounds that up-regulate the cellular production of interferon might have therapeutic uses for fighting viruses that fail to activate interferon by the host immune system. The purpose of this project was to dissect the mechanisms of induction of type I IFN by compound 97 which was discovered by Dr. Shenghua Zhou in the laboratory of Dr. Robert W. Finberg. The upregulation of interferon mRNA and protein will be monitored by RT-PCR, the Nanostring technology, and a type I IFN bioassay, respectively. The ability of the compounds to lower cellular viral titers will be monitored for LCMV and recombinant VSV-GFP to observe the potential protection of interferon against these viruses. Cells derived from RIG-I, MDA-5, and TLR3 knockout mice were used.

Materials and Methods

Cell Culture

Human Dermal Fibroblast, neonatal (HDFn cells) cells were obtained from Invitrogen, and NCTC929 cells were originally derived from mouse fibroblast L929 cells. In a sterile hood, 4 mL of medium that contained 5% fetal bovine cell serum, penicillin and streptomycin, was added to a 50 ml tube before adding all cells from the sample (separate tubes for each sample). The tube was then centrifuged for 2 min at 1000 rpm, the supernatant was discarded, and the cell pellet was transferred to a flask by adding pre-warmed medium to the 50 mL tube. For cells to be transferred to T-25 flasks, 6 mL of medium was added, and for T-75 flasks, 12 mL of medium.

Bioassay for Mouse Type 1 IFNs

A bioassay was used to determine the bioactivity of interferon produced in cells exposed compound or positive / negative control. NCTC929 cells were cultured, then the number of cells were adjusted to 1×10^5 cells/mL in MEM 5% FCS/Pen-Strep. Using 96 wells plates, a 2-fold series dilution of the cell supernatant samples that were collected from compound-treated cells was performed, with a final volume of 100 uL per well. 100 uL of NCTC929 cells added to each well. After overnight incubation of the cells at 37°C, VSV virus was diluted to 1×10^3 pfu/mL. The old medium was aspirated and 200 uL of diluted VSV was used to challenge NCTC929 cells. Cells were incubated with VSV for 48 hours. Each well of cells was observed under an inverted microscope to determine the number of cells that were protected against the virus. The well that had 50% of cells protected from the VSV challenge was the threshold at which the tested sample can protect NCTC929 cells from VSV. This dilution of sample is defined as 1

U/ml of bioactivity of type 1 interferons. The level of the bioactivity of type I IFN was calculated by the following formula: The highest dilution of sample that protected 50% of NCTC929 cells from VSV challenge x dilution factor.

RNA Isolation for RT-PCR and Nanostring Assays

After cells were treated with various doses of interferon-producing compounds, the cells were harvested and lysed with RNeasy Lysis Buffer. The cell homogenate was placed in a gDNA Eliminator spin column in a 2 ml collection tube, and centrifuged for 30s at >10,000 rpm. The gDNA column was discarded, and 350 uL of 70% ethanol was added to the flow-through (containing RNA), and mixed by pipetting. 700 uL of the sample was transferred to an RNeasy spin column in a 2 mL collection tube, and centrifuged for 15 s at >10,000 rpm. The column flow-through was discarded, and 700 uL of Buffer RW1 was added to the RNeasy spin column. This was centrifuged for 15s at >10,000 rpm and the flow-through was discarded. 500 uL buffer RPE was added to the spin column, centrifuged for 15s at >10,000 rpm and the flow-through discarded. 500 uL of buffer RPE was added once more, and the column was centrifuged for 2 min at >10,000 rpm. The RNeasy spin column was transferred to a new 1.5 mL collection tube, and ~40 uL of RNase-free water was added, and the column was centrifuged for 1 min at >10,000 rpm. The RNA collected in the flow-through was used for RT-PCR and Nanostring assays.

cDNA Synthesis

In one 0.2 mL thin-walled PCR tube per sample on ice, 1 uL of Oligo(dT)₂₀ primer, 1 uL of annealing buffer, up to 6 uL of RNA, and RNase/DNase free water were mixed together in

equal amounts per sample and incubated in a thermal cycler at 65°C for 5 min. The reaction was then placed on ice for 1 min immediately following incubation, and centrifuged briefly to collect the reaction solution. 10 uL of 2x First-strand Rxn Mix and 2 uL of Superscript III/RNase Out Enzyme mix were added to the tubes, they were vortexed to mix, and then briefly centrifuged to collect the solution. The tubes were then placed in the PCR machine for 50 min at 50°C before being terminated at 85°C for 5 min, and placed on ice. Using Nanodrop technology, the amount of cDNA per sample was determined and 200 ng of cDNA was used per sample for RT-PCR.

RT-PCR Assay

In 1 mL tubes, Taq, Primer, and dH₂O (see table below) were mixed, then ~47 uL of this mixture was added to each PCR tube. These amounts varied with each trial. cDNA (usually 3 µL) was added afterwards for a final reaction volume of 50 uL per tube. The PCR tubes were then placed in a PCR machine at 95°C for 6 min before beginning the cycles of temperatures (see table below) for ~32 cycles, and ending with 72°C for 7 min. The tubes were then stored at 4°C. The tubes were then mixed with 3 uL of dye, and run on an electrophoresis gel. This gel was photographed under UV light.

Nanostring Procedure

Nanostring technology was invented by Dr. Krassen Dimitrov (Nanostring Technologies, Seattle, Washington) (Geiss et al., 2008) and uses an “nCounter Analysis System” to measure a multiplex digital signal of gene expression inside a cell. The signal is highly precise and sensitive, and can detect one copy of a transcript per cell (Amit et al., 2009). The procedure can

perform single-molecule imaging to detect and count hundreds of transcripts in a single reaction. The technique analyses fewer transcripts than microarrays, but is more accurate and sensitive.

For this project, the procedure was performed 12 samples at a time, as this was the maximum that could be read in one run. First, a master mix of 130 uL of the Reporter CodeSet and 130 uL of the hybridization buffer was mixed, then 20 uL was added to each tube along with 5 uL of each sample RNA. The thermocycler was preheated to 65°C, and 5 uL of Capture ProbeSet was added to each sample before it was placed in the thermocycler where it was incubated for a minimum of 12 hours. The samples underwent some post-hybridization processing with the nCounter Prep Station, and then data was collected.

Results

Bioassay and VSV-GFP Experiments (Cell Survival and Viral Levels)

Human fibroblast cells were used to determine the bioactivity of type 1 interferon after treatment of the cells with compound 97 or its analogs 768 and 795, to validate our lab's previous finding (shown in Figure 4). A bioassay that determines the ability of a compound to protect viral-infected cells *in vitro* by measuring surviving cells was performed using supernatant samples with compound concentrations ranging from 0.0025 μM to 12 μM (**Figure-8**). All three samples showed no bioactivity for the lower concentrations, but compound 768 started to show bioactivity at 0.75 μM , and increased to 256 U/ml at the 12 μM concentration. Compound 97 and 795 started to show bioactivity at 3 μM , and increased to 64 U/ml and 128 U/ml, respectively. DMSO served as a negative control, and did not show any bioactivity at the highest concentration.

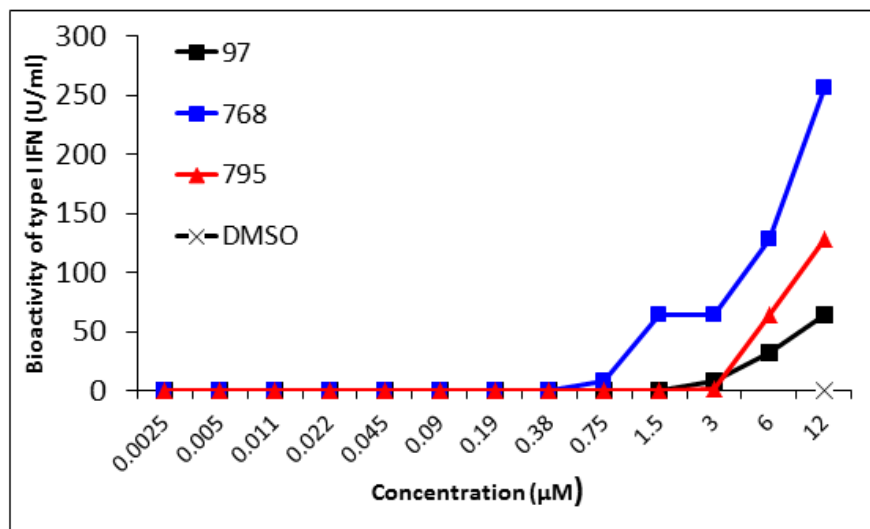


Figure-8: Induction of Type 1 Interferon in Human Fibroblast Cells by Compound 97 and its Analogs. Cells were treated with compound or control for 18 hours, then supernatants were used to perform a type 1 interferon bioassay. DMSO was used as a negative control.

Next, human fibroblast cells were treated with compound 97 or its analogs at concentrations varying from 3 μM to 50 μM for 18 hours, and then infected with VSV-GFP. Viral concentration was measured by flow cytometry at 24 hours post infection, and quantified as a value of mean fluorescence intensity (MFI) (**Figure-9**). A range of compound concentrations were used to determine if the compounds could inhibit VSV-GFP replication and at which concentrations.

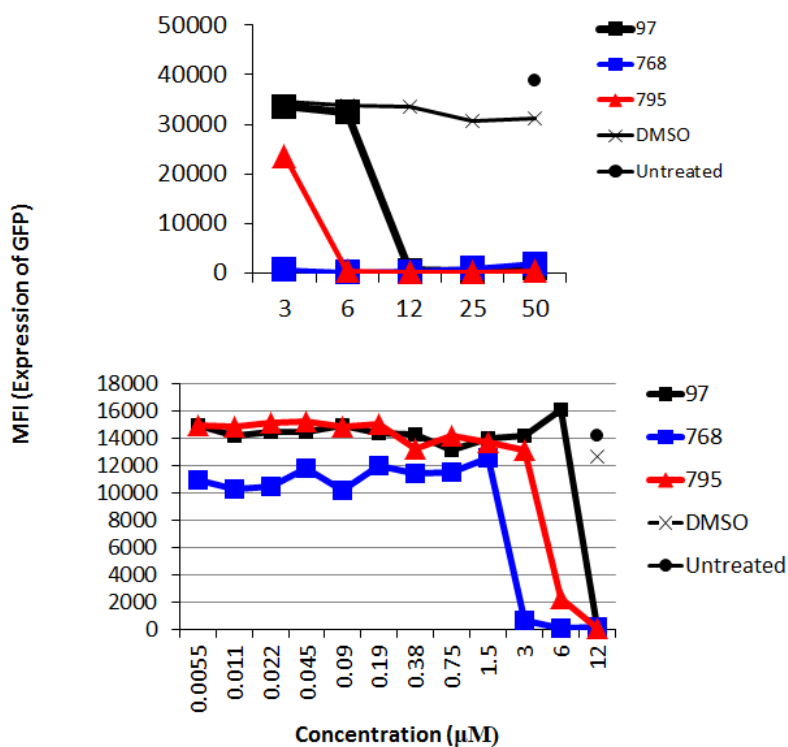


Figure-9: Inhibition of VSV-GFP Replication by Compounds in Human Fibroblast Cells. Cells were treated with compound or control for 18 hours, then incubated with VSV-GFP for 24 hours. The viral levels were measured by flow cytometry. DMSO and untreated were used as negative controls.

Compound 97 was able to inhibit viral production at concentrations higher than 12 μM . Compound 795 inhibited viral production at concentrations 6 μM and higher. Since compound 768 inhibited viral production at 3-50 μM (upper panel), we tested a wider range of compound

concentrations near the lower end, ranging from 0.0055 μM to 12 μM . Our data showed that 3 μM was the cutoff for compound 768's ability to inhibit the VSV-GFP production (lower panel).

Next, the level of bioactivity of type 1 interferon produced by the parent compound was measured at different time points using a bioassay on human monocyte-derived dendritic cells (**Figure-10**). The cells were treated with medium, DMSO or compound 97 at 50 μM or 100 μM , and the supernatant was collected after 7, 24, and 48 hours. These samples were used to perform a bioassay using VSV to determine the bioactivity of type 1 interferon. For both concentrations tested for compound 97, the highest bioactivity occurred at 24 hours, and was higher for the 100 μM concentration sample. The bioactivity decreased after 48 hours in both samples. This was compared to the medium and DMSO samples which did not show any bioactivity. Not shown here is the positive control, SeV, which induced interferon levels of about 2000 U/ml in cells.

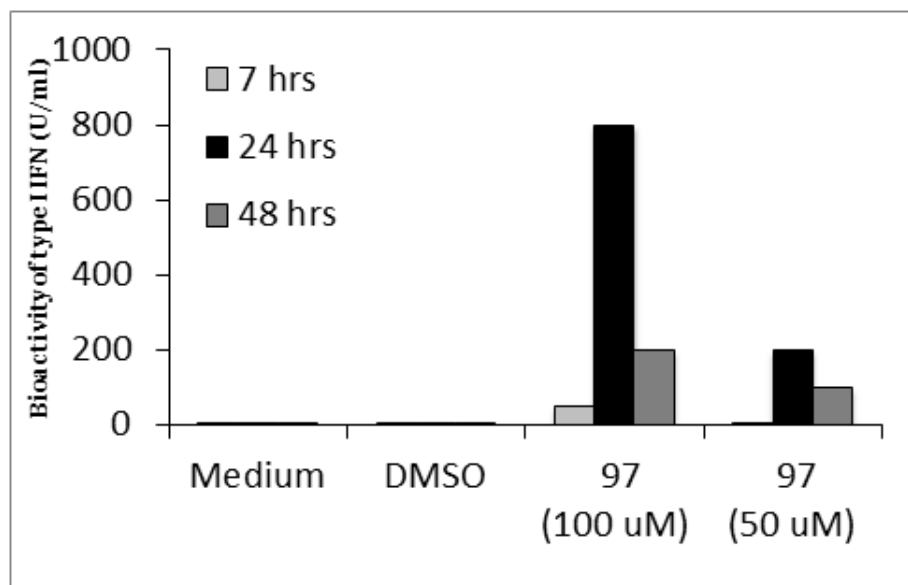


Figure-10: Stimulation of Type 1 Interferon Production in Human Monocyte-Derived Dendritic Cells by Compound 97. Cells were treated with compound 97 and the supernatant was collected after 7, 24 and 48 hours. A type 1 interferon bioassay was performed using these samples. Medium and DMSO were used as negative controls.

RT-PCR Experiments

In order to determine which signaling pathway is important for the induction data of type 1 interferon by compound 97, various knockout (KO) mouse embryonic fibroblast (MEF) cells were used with targets previously shown to regulate interferon production. RT-PCR was performed to measure the mRNA levels of IFN- β , RIG-I, and an internal loading/housekeeping gene GAPDH (**Figure-11**). RIG-I KO MEFs were chosen due to our lab's previous data suggesting this protein might be required for IFN production.

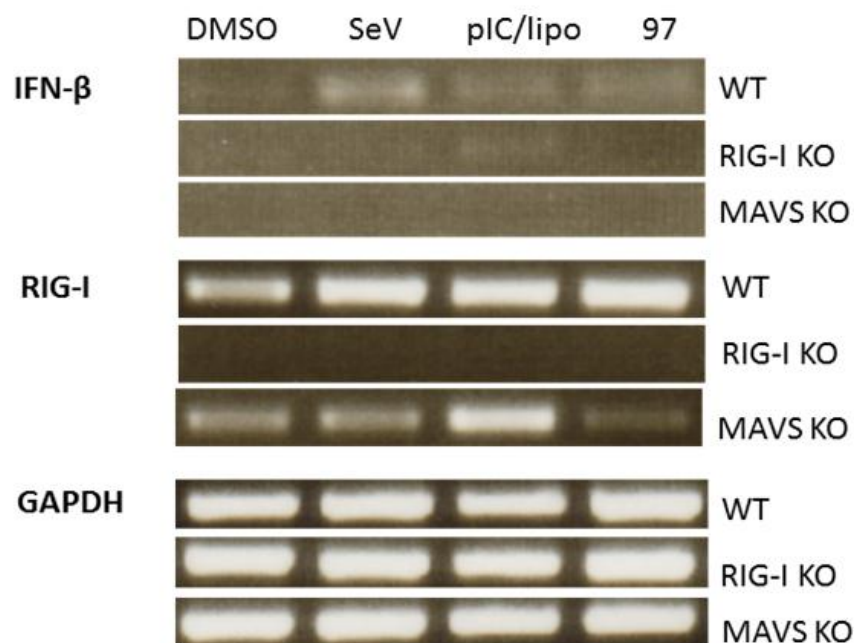


Figure-11: RT-PCR of IFN- β , RIG-I, and GAPDH in WT, RIG-I KO, and MAVS KO MEF Cells. mRNA levels of IFN- β , RIG-I and GAPDH were evaluated in cells treated with negative control DMSO, positive controls SeV and pIC/lipo, and compound 97.

Three types of MEF cells were used: wild type, RIG-I KO, and MAVS KO. Each cell line was treated with DMSO, SeV, pIC/lipo, or compound 97. Poly I:C also acts as a positive control

since it induces interferon production via RIG-I, MDA5, and TRL3. It was used because SeV is a weak positive control in RIG-I KO MEFs. Compound 97 induced the production of IFN- β mRNA in wild type cells, but not in RIG-I KO and MAVS KO cells. RIG-I mRNA production was high in wild type cells treated with SeV, pIC/lipo, and compound 97. A decrease in mRNA levels of RIG-I was present for the MAVS KO cells treated with compound 97 compared to the controls. GAPDH was used as load marker. The data indicate that RIG-I and MAVS likely are required for the production of IFN- β mRNA.

RT-PCR was also performed on wild type and TBK1 KO MEF cells to measure the mRNA levels of IFN- β , MDA5, and GAPDH. The cells lines were treated with DMSO, SeV, poly I:C, or compound 97 (**Figure-12**).

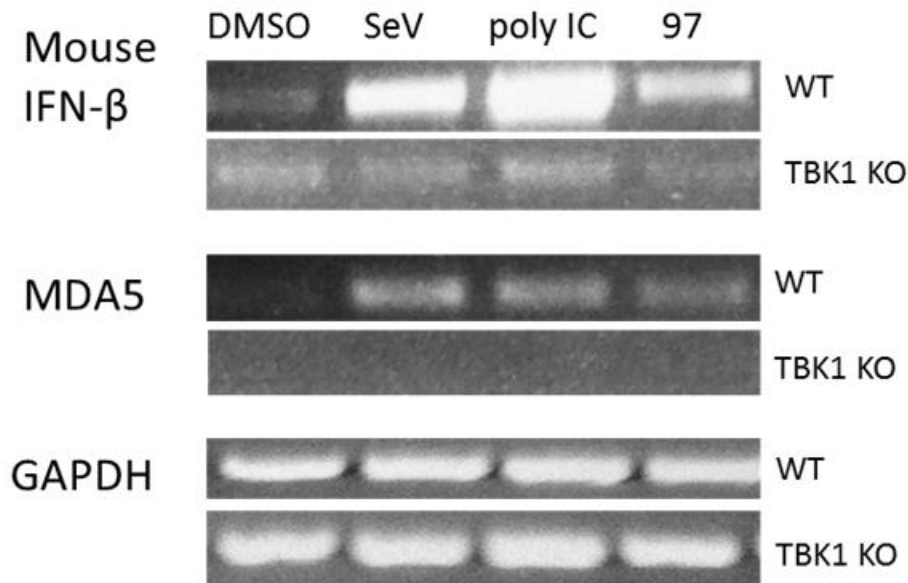


Figure-12: RT-PCR of IFN- β , MDA-5, and GAPDH in WT and TBK1 KO MEF Cells. mRNA levels of IFN- β , MDA5 and GAPDH were visualized after treated with DMSO, SeV, poly IC, or compound 97 for 18-24 hour.

The cells treated with compound 97 show increased IFN- β mRNA in wild type cells compared to DMSO, while TBK1 KO cells show no increase in IFN- β mRNA levels. This was also the same

for MDA5 mRNA levels, where WT cells showed increased levels and TBK1 KO cells did not for any treatment. GAPDH was used as a loading control.

Nanostring Experiments

Nanostring technology (see Methods) uses an “nCounter Analysis System” to measure a multiplex digital signal of gene expression inside a cell. The signal is more precise and sensitive than a microarray, and can detect one copy of a transcript per cell (Amit et al., 2009). This technique was used to supplement the RT-PCR data. A nanostring analysis was done on 47 interferon inducible genes and 3 housekeeping genes in wild type and RIG-I KO MEFs. The cells were treated for 18–24 hours with DMSO, compound 97, SeV, or pIC/lipo, and then their RNA was extracted. The copies of several mRNAs related to IFN production were counted and normalized to the Hprt1 housekeeping gene. **Figure-13** shows the results for IFN- β 1, STAT1, and STAT2 mRNAs. In WT cells, compound 97 increased the levels of all 3 mRNAs relative to DMSO treated cells, but not in RIG1-KO cells. Positive control SeV increased both STAT mRNAs (but not IFN- β 1) in WT cells, and as expected not in RIG1-KO cells. pIC increased all 3 mRNAs in all cell lines.

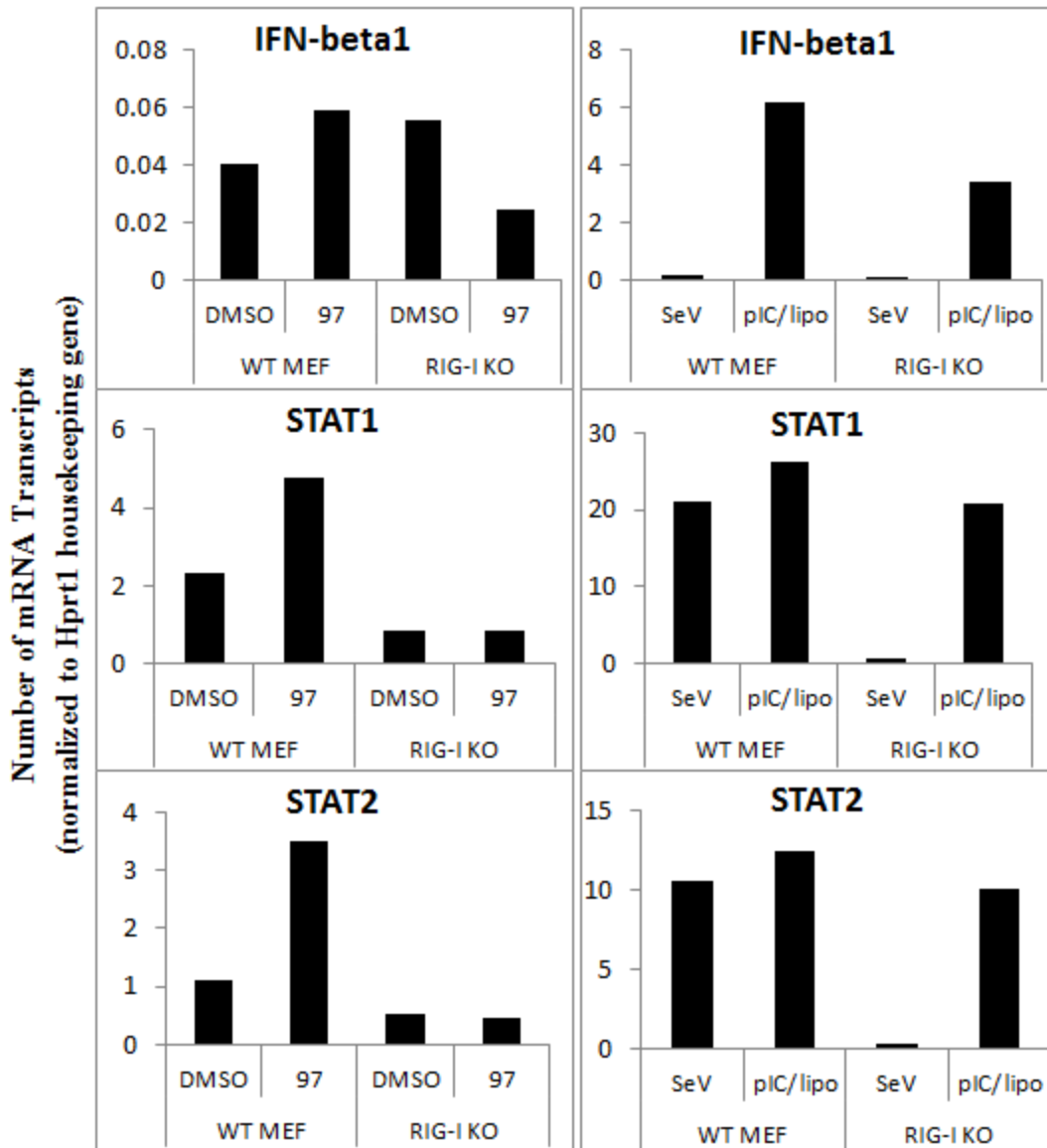


Figure-13: Summary of Nanostring Analysis of IFN- β 1, STAT1, and STAT2 mRNA Levels in WT and RIG-I KO MEFs Treated with DMSO, Compound 97, SeV, or pIC/lipo. mRNA transcripts of IFN- β 1, STAT1 and STAT2 were counted via a digital signal, and normalized to HPRT1.

mRNAs for IRF7, IRF3 and IFI205 were also analyzed by the nanostring method (Figure-14). These mRNAs were chosen due to their known roles as interferon response factors. The levels of IRF7 and IFI205 mRNA transcripts strongly increased in wild type MEFs treated

with compound 97 compared to DMSO, but not in RIG-I KO cells. IRF3 appeared to be unaffected by compound 97 in both WT and RIG-I KO MEFs.

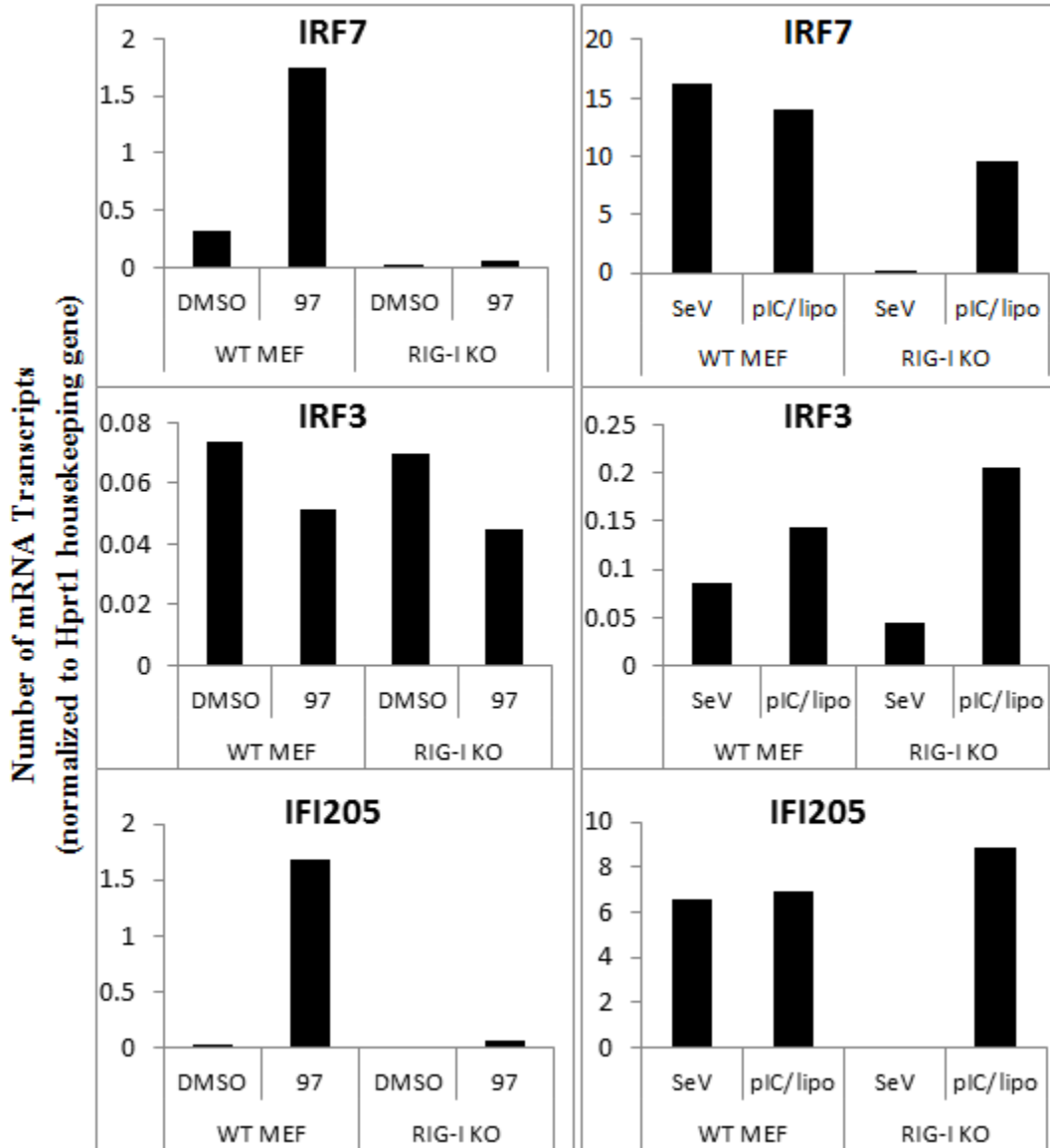


Figure-14: Nanostring Analysis of IRF7, IRF3, and IFI205 in WT and RIG-I KO MEFs Treated with DMSO, Compound 97, SeV or pIC/lipo. mRNA transcripts of IRF7, IRF3 and IFI205 were counted via a digital signal, and normalized to Hprt1.

The nanostring approach was also applied to RIG-I, MDA5, and Lgp2 (**Figure-15**). Lgp2 is also a RIG-I-like receptor and is required for RIG-I and MDA5 responses (Sato et al., 2010).

In WT cells, all three transcripts strongly increased using compound 97 (compared to DMSO), but not in RIG1-KO cells.

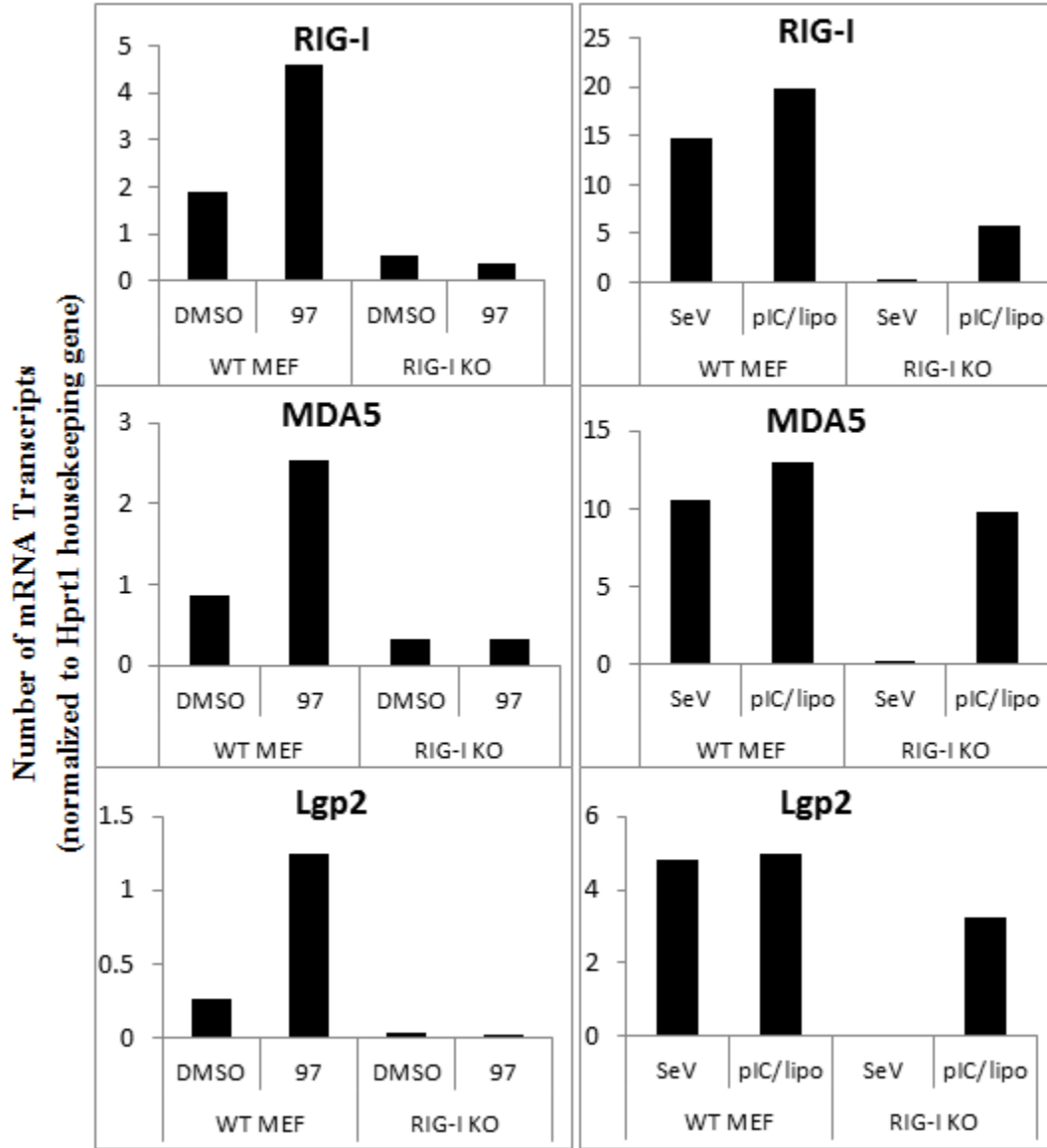


Figure-15: Nanostring Analysis of RIG1, MDA5, and Lgp2 in WT and RIG-I KO MEFs Treated with DMSO, Compound 97, SeV or pIC/lipo. mRNA transcripts of RIG-I, MDA5 and Lgp2 were counted via a digital signal, and normalized to Hprt1.

The mRNA levels of three chemokines were also counted using the nanostring procedure. The mRNAs for CCL5, CXCL10 and CXCL1 strongly increased in WT cells treated with compound 97 relative to DMSO-treated cells, but not in RIG1-KO cells (**Figure-16**).

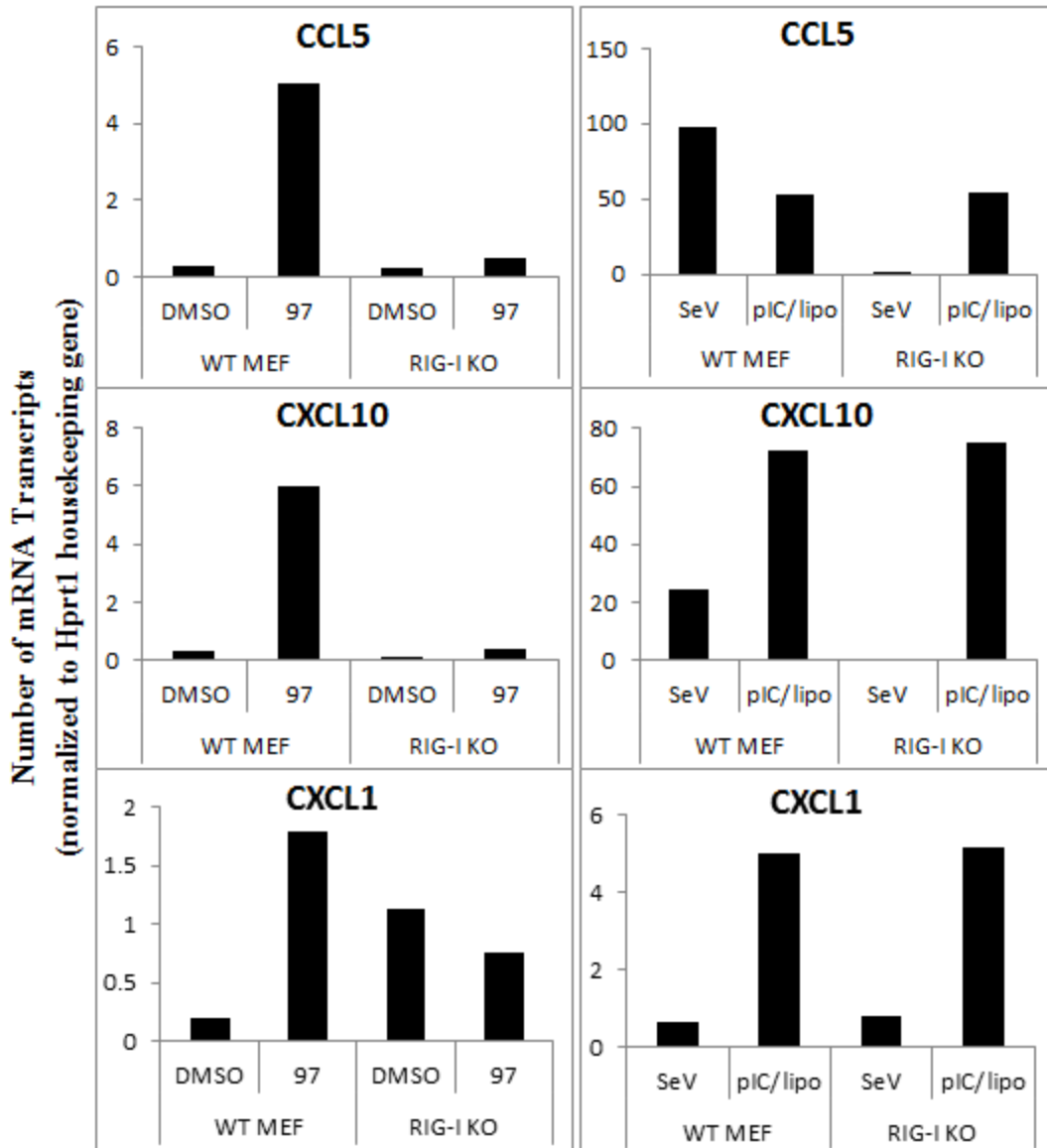


Figure-16: Nanostring Analysis of CCL5, CXCL10, and CXCL1 in WT and RIG-I KO MEFs Treated with DMSO, Compound 97, SeV, or pIC/lipo. mRNA transcripts of CCL5, CXCL10 and CXCL1 were digitally counted and normalized to Hprt1.

Discussion

At the beginning of the compound investigation, compound 97 was shown to induce interferon production in various types of cells, as measured by the increased IFN- β 1 mRNA production, increased survival of cells infected with Lymphocytic Choriomenigitus Virus (LCMV), and lower levels of residual virus. Compound 97 was chosen to continue the studies when the initial test of Interferon- β production occurred and narrowed down the two choices to compound 97 and compound p21, both inducing high amounts of interferon production in human monocytes cells (Figure-2) but eliminating p21 (Figure-3). Compound 6387 was also not considered for moving forward because while the compound induced interferon production in mouse fibroblast cells, it did not in human fibroblast cells.

Human monocytes cells, which are precursors to dendritic cells, were treated with compound 97 and its analogs in order to determine the best conformation of the compound to be used for interferon production (Figure-4). All three compounds were able to lower the residual amounts of virus in human fibroblast cells (Figure-5).

This project also investigated the specific pathway that compound 97 is inducing to produce the interferon. Previous studies identified a variety of pathways known to up-regulate interferon production (Figure-1) which presented targets for our analysis. Observing the amount of mRNA transcripts via RT-PCR (Figure-6), MDA5 was shown to be important in interferon production induced by these compounds, as indicated by the strong signals from the compounds and its analogs compared to the positive control Sendai Virus (SeV). The data indicated that the compound could be inducing interferon through the two major signaling pathways (Figure-7). The goal was to determine if one of these pathways was the primary pathway.

Compound 97 and its analogs were tested in human fibroblast cells in a bioassay to demonstrate the levels of interferon each compound induced in these cells (Figure-8). Comparing these levels to the negative control, DMSO, this figure shows that the compounds induce significant levels of interferon production that could possibly aid cells in defense against viral infection. To test this idea, these human fibroblast cells treated with these compounds once again were exposed to VSV-GFP. The upper portion of Figure 9 shows that these compounds were able to protect against viral infection compared to the negative controls. In order to get a clearer picture of the effect of the analogs, especially 768, this experiment was repeated with more dilutions (lower portion of Figure 9). This data confirms that cells treated with these compounds were better able to protect themselves against viral infection which could be used against other viruses.

In order to establish a baseline for comparison, the rest of the experiments used only compound 97, as it is the parent construct. Human monocyte-derived dendritic cells were treated with compound 97 at two different concentrations, 100 μ M and 50 μ M, in a bioassay (Figure 10) to determine whether the compound could also induce interferon production in these cells as well as the human fibroblast cells. These dendritic cells are important in innate immunity, as upon initial infection of a virus they are the major interferon producer used to recruit other cells to the site of infection. This figure also indicates at which time point the levels of interferon production are most significant, as well as which concentration of the compound was more effective.

Based on the data collected by Finberg's lab, it was shown that Compound 97 was able to induce significant levels of interferon production. RIG-I and MDA5 were the proteins of a major pathway thought to induce interferon production, and were tested in Figures-11 and-12 in mouse

embryonic fibroblast cells (MEFs). RIG-I was tested first (Figure-11) by knocking out (RIG-I KO) the gene that produces this protein, and the cells were then treated with the Sendai Virus (SeV), poly I:C, DMSO, and compound 97. SeV was used as a positive control since it is extremely sensitive and induces large amounts of interferon production through RIG-I signaling. Poly I:C is also a positive control since it induces interferon production via MDA5 and TRL3. DMSO displayed the basal levels of mRNA production as the negative control. The RT-PCR results show that RIG-I and MAVS are important for inducing interferon production by compound 97. When these proteins are nonfunctional, the IFN- β mRNA levels decreased compared to wild type cells treated with compound 97 although the strength of these signals is thought to be due to the time point when these were collected may not have been optimal. This decrease indicates that when RIG-I or MAVS are nonfunctional, the compound is unable to stimulate a pathway to create interferon. When wild type cells are treated with compound 97, mRNA levels of RIG-I also increased, indicating that the pathway has been activated. In MAVS KO cells, RIG-I mRNA levels are low, indicating that the removal of MAVS prevents a feedback loop to increase RIG-I production even though it is being activated by compound 97.

Farther down in this pathway is TBK1. This protein is involved in all major type 1 interferon production pathways starting from RIG-I/MDA5 and TLRs. When TBK1 is knocked out, compound 97 is unable to make high levels of IFN- β and MDA5 (Figure-12). mRNA levels of MDA5 are increased when wild type cells are treated with compound 97 but nonexistent in TBK1 cells. This shows that TBK1 is also important in the induction of interferon production by compound 97 and that without TBK1, the pathway cannot be completed to induce type 1 interferon production and stimulate the upregulation of other pathway components.

A new technology called Nanostring was used to demonstrate the importance of RIG-I in interferon production. This procedure is a highly sensitive, accurate measure of cellular mRNA levels that uses individual molecule fluorescence and digital readouts. This approach was used to investigate the levels of mRNAs previously shown to participate in interferon production. Figures 13-16 show the number of mRNA transcripts for 12 genes related to interferon production in wild type MEFs or RIG-I KO MEFs. The cells were treated with DMSO, compound 97, SeV, or pIC/lipo. A housekeeper gene, Hprt1, was used to normalize the data for comparison between the genes and between wild type and knockout cell lines. The data showed that in WT cells, compound 97 increased the levels of mRNAs for IFN- β 1, STAT1, STAT2, IRF7, IFI205, RIG-I, MDA5, Lpg2, CCL5, CXCL10 and CXCL1, relative to DMSO-treated cells, and that all these transcripts did not increase in RIG1-KO cells. This shows that without RIG-I, the upregulation of the interferon production and signaling pathways does not occur. One exception was observed in this data. Number of IRF3 transcripts appeared to be unaffected by compound 97 in both wild type and RIG-I KO cells. It has been demonstrated that IRF3 is constitutively expressed and its expression is not regulated by interferon responses.

MDA5 is similar to RIG-I which means they both could be involved in compound 97 activation of the type 1 interferon production pathway. In order to determine its role, a future experiment may include using a silencer sequence (siRNA) on the gene that produces MDA5 in RIG-I KO MEFs and observing its abilities against virus infection. Levels of interferon and mRNA can be measured and compared to normal RIG-I KO MEFs and wild type MEFs. With both MDA5 and RIG-I unable to activate the pathway, this could show if the TLRs are activated by compound 97. To further confirm the role of RIG-I in MEFs, another future experiment is to attempt to rescue the effect of having and RIG-I KO by complementing MEF cells with a

plasmid containing a new RIG-I gene and observing whether or not the cells return to wild type behavior. If the wild type levels return, this would indicate that the knockout of RIG-I was involved in the cause of the decreased levels of interferon and support the idea that the RIG-I pathway is a major pathway that compound 97 uses to induce type 1 interferon production.

These RIG-I KO cells were mouse cells, not human. In order to determine if RIG-I is just as important in human cells, another siRNA knockdown would have to be performed on the RIG-I and MDA5 genes, as well as genes that activate the other half of the hypothesized pathways, such as the TLRs. Similar knockdown tests would be performed to see if compound 97 works in the same way in human cells. These results could present the medical field with a solution to fighting viruses that are able to circumvent the innate immunity and cause serious damage to the body.

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