THE ROLE OF NS5A IN CHRONIC HCV INFECTION

by

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Abstract

Hepatitis C is a viral disease that affects the liver cells of the infected individual, resulting in inflammation and frequent liver failure. Currently 150-200 million people worldwide are infected with Hepatitis C and 85% of these incidences have developed into a chronic condition. The causative agent for Hepatitis C is HCV (hepatitis C virus): an enveloped, positive single-stranded RNA virus, and a member of the *Flaviviridae* family. The RNA genome possesses ten structural and nonstructural proteins, notably Nonstructural Protein 5A (NS5A), the center of our research. When HCV infects the host, a downstream activation of the antiviral response produces type I interferons and proinflammatory cytokines. By an unknown process, NS5A possesses the capability of inhibiting the expression of IFN-β. The goal of this project was to characterize the inhibition of the signaling pathway leading to antiviral gene expression. To better understand this interaction, we used a commercially available NS5A inhibitor, Daclatasvir, in combination with a mutant of NS5A, NS5A-10A, known to inhibit the IFN-β promoter. As expected, we observed a reduction in IFN-β expression in the presence of a mutated NS5A-10A. The level of IFN-β expression increased when Daclatasvir was added, validating the observation that NS5A is capable of inhibiting IFN-β gene expression.

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Introduction

Hepatitis C is a viral disease that affects the liver cells of the infected individual, resulting in inflammation and liver failure in close to 70% of patients. Currently an estimated 150-200 million people worldwide are infected with hepatitis C. In eighty-five percent of incidences, the infection results in a chronic disease (CDC). Hepatitis C increased following organ transplants and blood transfusions due to the lack of screening prior to 1989 (Feitelson). Today the major mode of transmission is via contaminated syringes. Patients with chronic hepatitis C consequently develop cirrhosis and liver cancer in twenty percent and five percent of cases, respectively. Unlike other forms of hepatitis, a preventative vaccine for hepatitis C virus has yet to reach the market. For this reason, research in developing a vaccine for hepatitis C virus is of vital importance.

The causative agent for hepatitis C is HCV (hepatitis C virus): an enveloped, positive single-stranded RNA virus, and a member of the *Flaviviridae* family. The RNA genome of HCV contains 9600 nucleotides and a single open reading frame, resulting in a single protein product via translation (Feitelson). This protein consists of both structural and nonstructural proteins produced once the single protein is cut and processed via cellular and viral proteases. The single protein product then produces ten smaller secondary protein products. These proteins include the structural proteins (core protein, envelope proteins E1 and E2), which function in assisting entry into the host cell and forming the nucleocapsid. In addition, the HCV genome contains seven nonstructural proteins namely NS1, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Dimitrova). The nonstructural proteins possess multiple enzymatic capabilities and assist in HCV replication. Progeny virions then bud out of the infected cell via the intracellular membranes, and then egress via the cellular secretary pathway before infecting another host cell (Von Hahn).

When Hepatitis C Virus infects a cell, the surface glycoproteins on HCV interacts with multiple cell-surface molecules on the host cell, including CD81, SR-BI, and CLDN1 (Von Hahn). CD81 in particular is responsible for the infectivity of hepatocytes. Structural protein E2 is responsible for interaction with the cell-surface molecules via receptor binding in the hypervariable region of the protein. Following the virus-receptor interactions at the cell surface, the virus particles finally enter the cell by receptor-mediated endocytosis (Von Hahn). The RNA genome is then released into the cytoplasm and translated at the rough ER, which gives rise to the polyprotein and subsequent mature proteins. In response to the infectivity, the host cell activates the antiviral response pathway. The HCV positive, single-stranded RNA genome produces a double stranded RNA molecule via replication. The initial step for cytokine response induction by RNA virus infection is activation of the cellular dsRNA receptor systems, Toll-like receptor 3, DexH(D) RNA helicase, and retinoic acid inducible gene 1 (RIG-I). TLR3 and RIG-I respond through adaptor proteins, TRIF and MAVS, which mediate the activation of IκB kinase, IKKe and TBK1, resulting in the activation of and nuclear translocation of IRF 3 (Seth). The C terminus of IRF-3 (interferon regulatory response factor) is phosphorylated, resulting in a conformational change that opens up the nuclear localization signal. Unveiling this signal results in phosphorylation and degradation of IκB, an inhibitor of the transcription factor, NF-κB. The accessible NF-κB is then translocated to the nucleus where it initiates transcription of many antiviral proteins, in particular type I interferons. NF-κB binds to the IFN-β promoter as a part of the enhancesome. In the nucleus, IRF3, NF-κB, and ATF-2 transcription factors activate type I IFN and proinflammatory cytokine gene expression (Seth). Upon synthesis, IFN-β then initiates several downstream signaling events involved in the innate immune response.

In order to continue to replicate in the host cell, viruses commonly adapt mechanisms that help to prevent activation of type I IFNs. Many nonstructural proteins of HCV are believed to function in interacting with this process. In particular, NS5A has demonstrated the ability to activate this interferon pathway at some intermediate step. Many of the activities of NS5A and other nonstructural proteins are speculated (Rhea); however, it is known that NS5A modulates the function of NS5B, an RNA-dependent RNA polymerase (Dimitrova). When mutated with an in-frame K insertion at amino acid position 2040, near the amino terminus of NS5A, designated NS5A-10A, protein replicates five fold greater than wild type NS5A (Rhea). In addition, work in our lab has shown that NS5A-10A renders a specific block in the antiviral signaling pathway that activates the IFN-β (Akkaraju). The NS3 protease in combination with its cofactor NS4A (NS3/4A) interferes with the infected cell's natural antiviral response (Kwong). The serine protease NS3/4A has been shown to inhibit IFN-β gene expression by inhibiting virus-induced activation of the IFN-β promoter. A well-studied phenomenon, the capability to inhibit the downstream production of interferons by NS3/4A has led to the production of multiple protease inhibitors that clinically reduce HCV RNA levels following oral administration (Kwong). The mechanism by which IFN-β expression is inhibited by NS5A-10A remains to be deciphered.

Methods

Cell Culture

Human embryonic kidney (293 HEK) cells grown in tissue culture were used for the duration of our experiments. HEK cells were incorporated with NS5A-10A protein to create the 293*NS5A-10A cell line. Both cell lines were grown in Dulbecco's Modified Eagle Medium in a 37^{o} C, 5% CO_2 tissue culture incubator between 10%-90% confluence in a T25 flask. Cells were passaged about twice a week or when the cell cultures appeared to have reached the appropriate density. For cell passage, the medium was aspirated from the flask. Cells were then washed with 1ml 1xPBS (Phosphate Buffered Saline). Next, cells were covered in 1 ml of 37^{o} C 0.05% Trypsin-EDTA for 2 minutes. A microscope verified that the cells had detached. The trypsinization terminated with the addition of 6 ml of DMEM. The cells and medium were titrated to disperse all clumps. Cells were counted using a microscope and flasks were then stored back in the incubator.

Luciferase Assay

Cells were first plated in the appropriate dishes using the same passaging procedure as described above. Cells were counted using a microscope to ensure accuracy. After 24 hours, the cells were transfected with the DNA of interest. Lyovec, lyophilized cationic lipid-based transfection reagent, was mixed with a combination of 100ng/well IFN-β and 50ng/well of RLCMV and 50μl of the solution was added to each well. After 24 hours, 10μl of Sendai virus was added to the designated well. Luciferase activity was measured using the Dual Luciferase Assay Kit (Promega). After another 24 hours, the medium from each well was removed and 250 μl of Passive Lysis Buffer (Promega) was added to each well for 15 minutes. The contents of the

well (lysates) were removed by pipetting up and down 3 times, dispensing into labeled tubes; 20μl of the lysates were dispersed into centrifuge tubes. For the luciferase assay, 50 μl of the Luciferase Assay Reagent (LAR) II was added to the centrifuge tube to generate a stabilized luminescence signal. The reaction is then quenched and the Renilla luciferase reaction is initiated by adding 50 μl of the Stop & Glo Reagent to the same tube. The resulting calculations are normalized by comparing infected cells (+SV) to the control cells (-SV).

MTT Assay

Cells were plated on a 96-well 1 day prior to experiment. MTT solution was prepared by weighing out 1 mg/ml of MTT and dissolving in Serum-Free Dulbecco's Modified Eagle's Medium. We added 100μl of solution to each well, leaving one blank as a control. Cells were incubated at 37^{o} C for 4 hours. MTT solution was removed from wells and 100μl of DMSO (Dimethyl sulfoxide) was added to the wells, and plates rocked back and forth for 5 minutes. Absorbance was measured at 540nm using a microtiter plate reader, including the blanks. The values were calculated by subtracting the value from the blank. Absorbance was plotted against number of cells/ml.

Western Blot

Every Western Blot was performed using a 9% polyacrylamide gel. The 10-lane fels were prepared one day in advance and refrigerated. The samples were prepared with SDS sample buffer and boiled for 5 minutes. The gels were loaded with 5μl of Precision Plus Protein Kaleidoscope (Bio Rad) as a marker, and 15μl of the prepared sample. Gels were immersed in Running Gel buffer and attached to a power source at 100V for 1 hour. While gels were running,

filter paper and PVDF membranes (Millipore) were soaked in methanol followed by Transfer Buffer. The gels were removed and the cassettes were prepared. Cassettes were prepared by layering sponge, filter paper, gel, nitrous membrane, filter paper, sponge in between the top and bottom of the plastic cassette. Transfers were run at 100V for 1 hour, allowing the proteins to transfer from the gel to the membrane. Blotto was prepared by mixing Tris Bugger Saline with Tween (TBST) with 5% milk powder. The membranes were removed and soaked in Blotto for 1 hour. Primary antibody was prepared by making a 1:1000 dilution of the primary antibody stock using Blotto. Membranes were left for 1 day in primary antibody. The next day, after approximately 14 hours, the membranes were removed from the primacy antibody and washed in Blotto for 5 intervals of 10 minutes each. Meanwhile, secondary antibodies were prepared by making a 1:10000 dilution of antibody stock in Blotto. Membranes were placed in their corresponding secondary antibody for 1 hour at room temperature. Membranes were washed again in Blotto for 5 intervals of 10 minutes each. Staining solution was prepared by combining 10ml AP buffer with 66μl NBT solution and 33μl BCIP solution. Membranes were removed from Blotto and washed with Tris Buffered Saline. Membranes were individually soaked in staining solution until bands appeared (approximately 5-45 minutes). Membranes were allowed to dry and were then analyzed.

Results

NS5A Present in Samples

Before conducting experiments to study this protein, we wanted to ensure that our 293*NS5A-10A cell line expressed the NS5A-10A protein. We performed a Western Blot experiment using both the 293 HEK control cell line and 293*NS5A-10A cell line with antibodies targeting NS5A protein. We used both infected and uninfected samples from both cell lines. We observed a protein band in the 293*NS5A-10A cell line, but we did not observe a protein band in the 293 HEK control cell line (Fig. 1). We used a Precision Plus protein ladder to ensure that the band we obtained corresponded in size with the NS5A protein. This observation confirmed that our 293*NS5A-10A cell line expressed the NS5A-10A protein, and the control cell line did not. We concluded that we could use this cell line to conduct experiments about NS5A-10A.

NS5A inhibits Sendai virus-infection induced IFN-β Promoter Activation

While we knew that NS5A-10A interfered with the cellular antiviral response, we wanted to demonstrate that NSA-10A specifically interacted with the ability to activate the IFN-β promoter. We performed a luciferase assay using both the 293 HEK control cell line and the 293*NS5A-10A cell line after infecting our cells with Sendai virus. Our luciferase assay indicated that the expression of NS5A-10A reduced the activation of the IFN-β promoter, as shown by the decreased luminescence (Fig. 2). Cell lines expressing a protein can sometimes change their phenotype in response to the long-term expression of a foreign protein. To show that the inhibition of the IFN-β promoter was not a non-specific result of long-term high-level expression of the protein, we transiently expressed NS5A-10A in 293 HEK cells by transfecting

the cells with an expression vector containing the gene for the NS5A-10A protein. We performed another luciferase assay using 293 HEK control cells, the 293*NS5A-10A cell line, and 293 HEK cells transfected with NS5A-10A. We demonstrated that the presence of NS5A-10A protein, in both the 293*NS5A-10A cell line and the transfected samples, reduced IFN-β promoter activation (Fig. 3). Our results confirm that NS5A-10A rendered a block in the antiviral pathway. Specifically, our results signify that NS5A-10A interacts with and hinders the activation of the IFN-β promoter.

Effect of NS5A Mutants on Gene Expression

After Hepatitis C Virus infects an individual, the NS5A protein can naturally mutate into new forms. For our experiment we obtained the NS5A Wild Type, the NS5A-10A mutant, and the NS5A-H27 mutant. Our goal was to investigate the interaction of these mutants with the antiviral response pathway. Specifically, we wanted to determine the effect of these NS5A mutants on the cell's ability to activate the IFN-β promoter. We transfected our 293 HEK cell line with NS5A Wild Type (WT), NS5A-10A, or NS5A-H27. Forty-eight hours after transfection, we performed a luciferase assay with our three transfected samples and the 293 HEK control cell line (Fig. 4). After infection with the Sendai virus, the cells containing NS5A-10A reduced the IFN-β promoter activation. In contrast, neither the NS5A Wild Type nor the NS5A-H27 reduced the IFN-β promoter activation (Fig. 4).

Daclatasvir Inhibits NS5A

For our next experiment, we wanted to assess the level of IFN-β promoter activation after inhibiting NS5A protein. We predicted that if we inhibit NS5A-10A by using a known inhibitor

called Daclatasvir, then this would allow for virus-induced activation of the IFN-β promoter. We performed a serial dilution of the Daclatasvir drug, and then performed a Luciferase assay using both the 293 HEK control cell line and the 293*NS5A-10A cell line treated with varying amounts of Daclatasvir (Fig. 5). Our results demonstrate how NS5A-10A untreated with Daclatasvir reduced the amount of IFN-β promoter activation in comparison to the 293 HEK cells. However, a gradual increase in the amount of Daclatasvir on the cells resulted in a gradual increase in IFN-β promoter activation. The Daclatasir inhibited the NS5A in a dose dependent manner, which thus reduced the inhibition of IFN-β promoter activation, thus resulting in a promotion of the IFN-β (Fig. 5).

Toxicity of Daclatasvir

When treating our cells with Daclatasvir, we wanted to eliminate the potential variable of toxicity of Daclatasvir on the cell line. This would allow us to conclude that the effect of Daclatasvir on IFN-β promoter activation is a result of inhibiting NS5A and now an interaction of Daclatasvir on cell survival. In order to assess the toxicity of Daclatasvir on our cells, we performed an MTT assay on our 293 HEK control cell line (Fig. 6) and our 293*NS5A-10A cell line (Fig. 7). The relative fold in cell population did not decrease with increasing concentration of Daclatasvir in neither cell line. Therefore, we determined that the Daclatasvir was not harmful to our cells (Fig. 6 and 7).

Figures

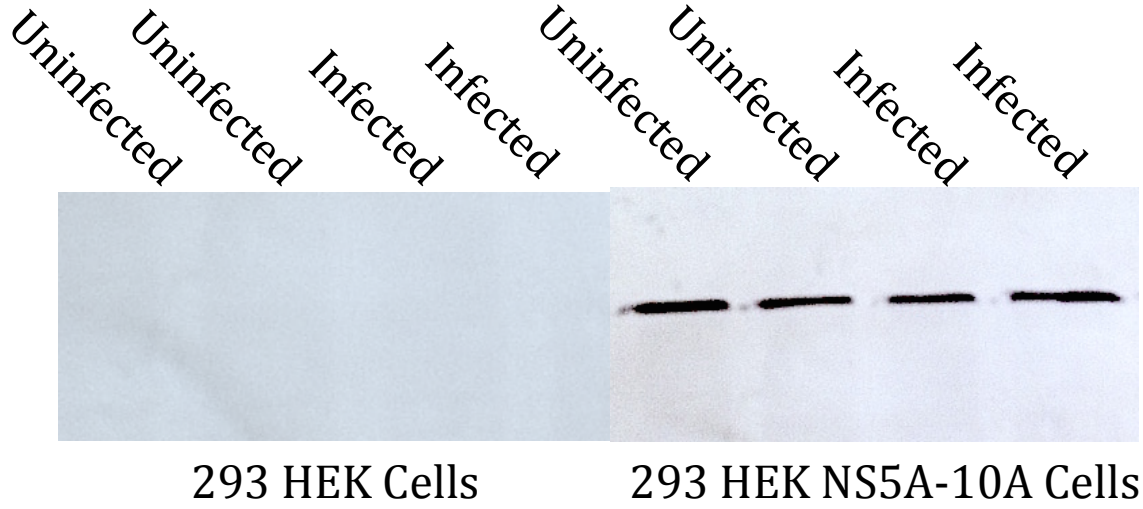

Figure 1: Western Blot Experiment comparing 293 HEK and 293 HEK NS5A-10A cells with antibodies targeting NS5A protein

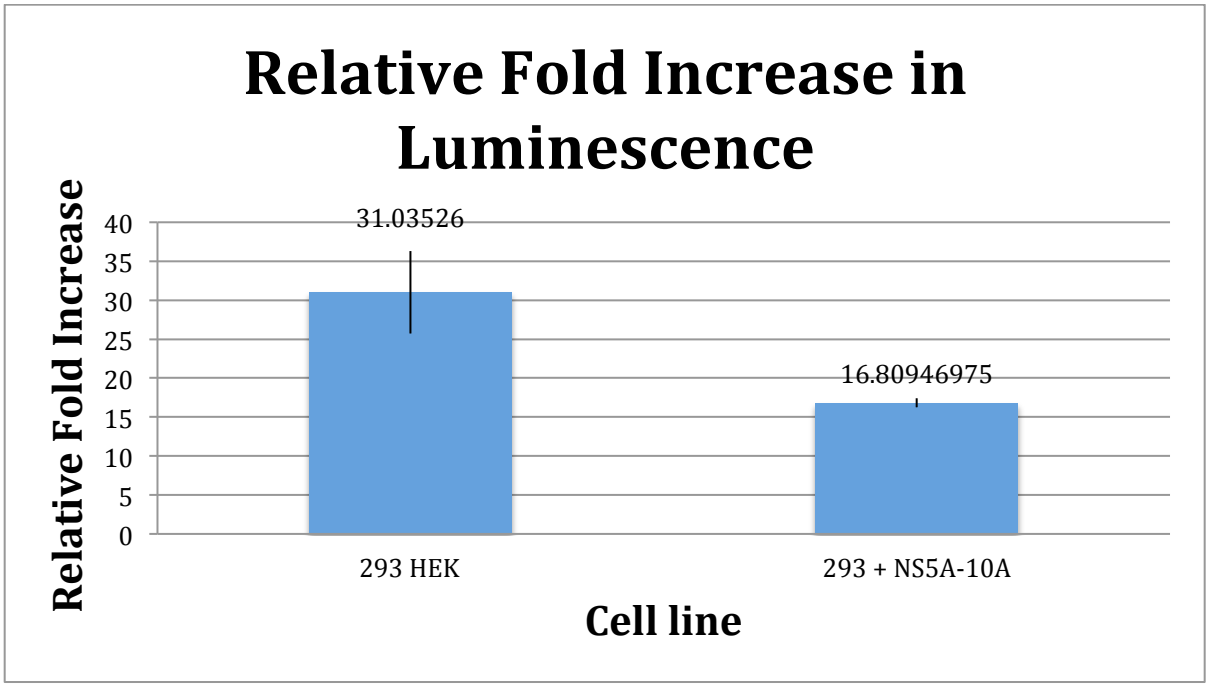

Figure 2: Luciferase Assay comparing the level of SV-induced IFN-β promoter activation in 293 HEK control cell line and 293*NS5A-10A cell line.

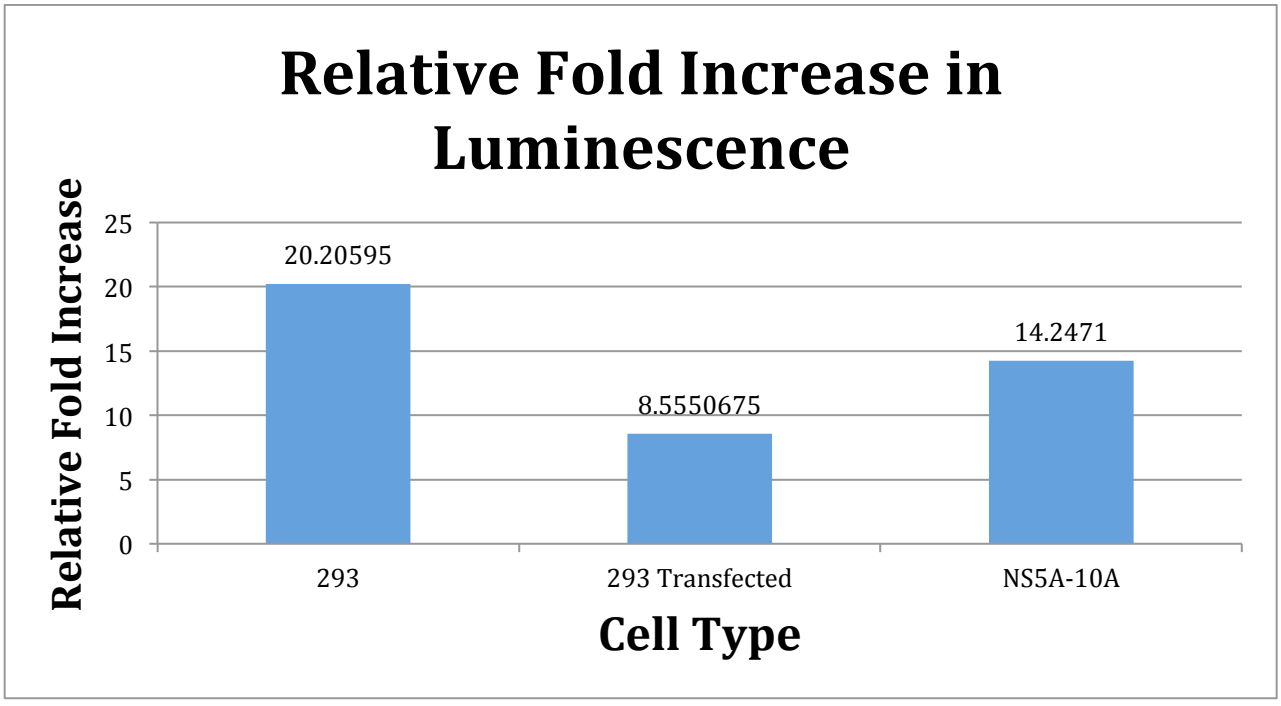

Figure 3: Luciferase Assay comparing the level of SV-induced IFN-β promoter activation in 293 HEK control cell line, 293 HEK transfected with NS5A-10A, and 293*NS5A-10A cell line.

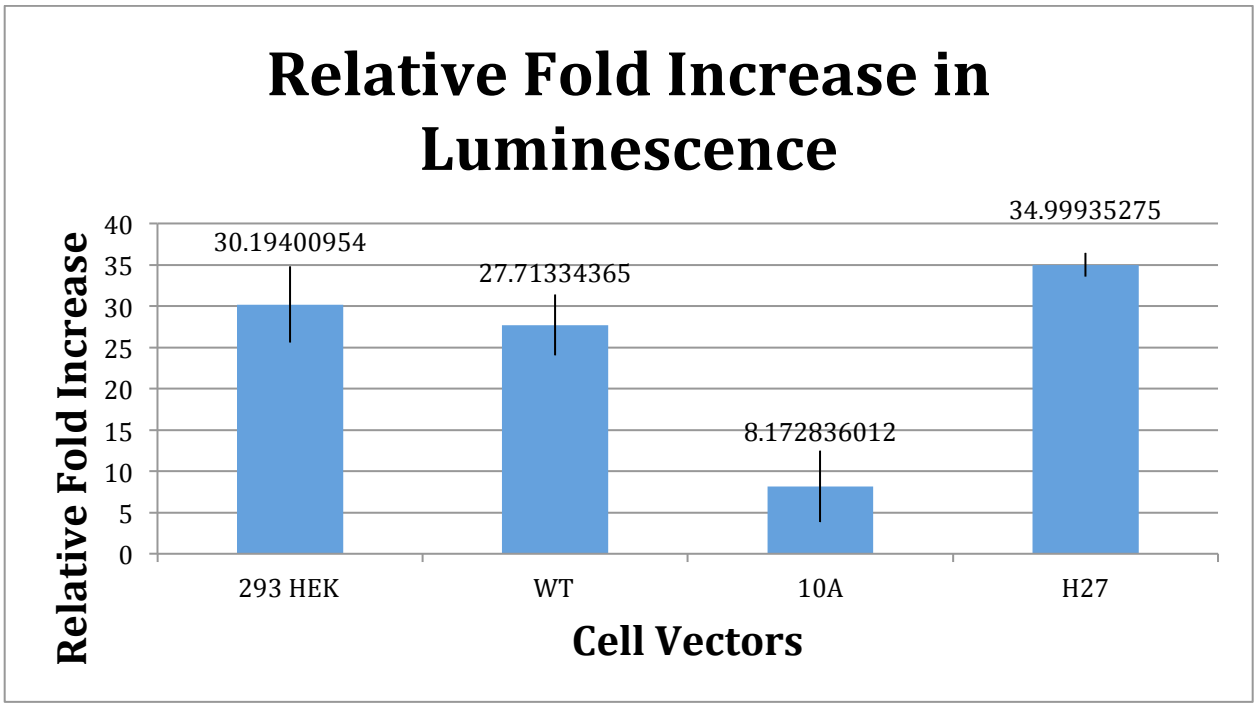

Figure 4: Luciferase Assay comparing the level of SV-induced IFN-β promoter activation in 293 HEK control cell line, 293 transfected with NS5A Wild Type (WT), 293 transfected with NS5A-10A (10A), or 293 transfected with NS5A-H27 (H27).

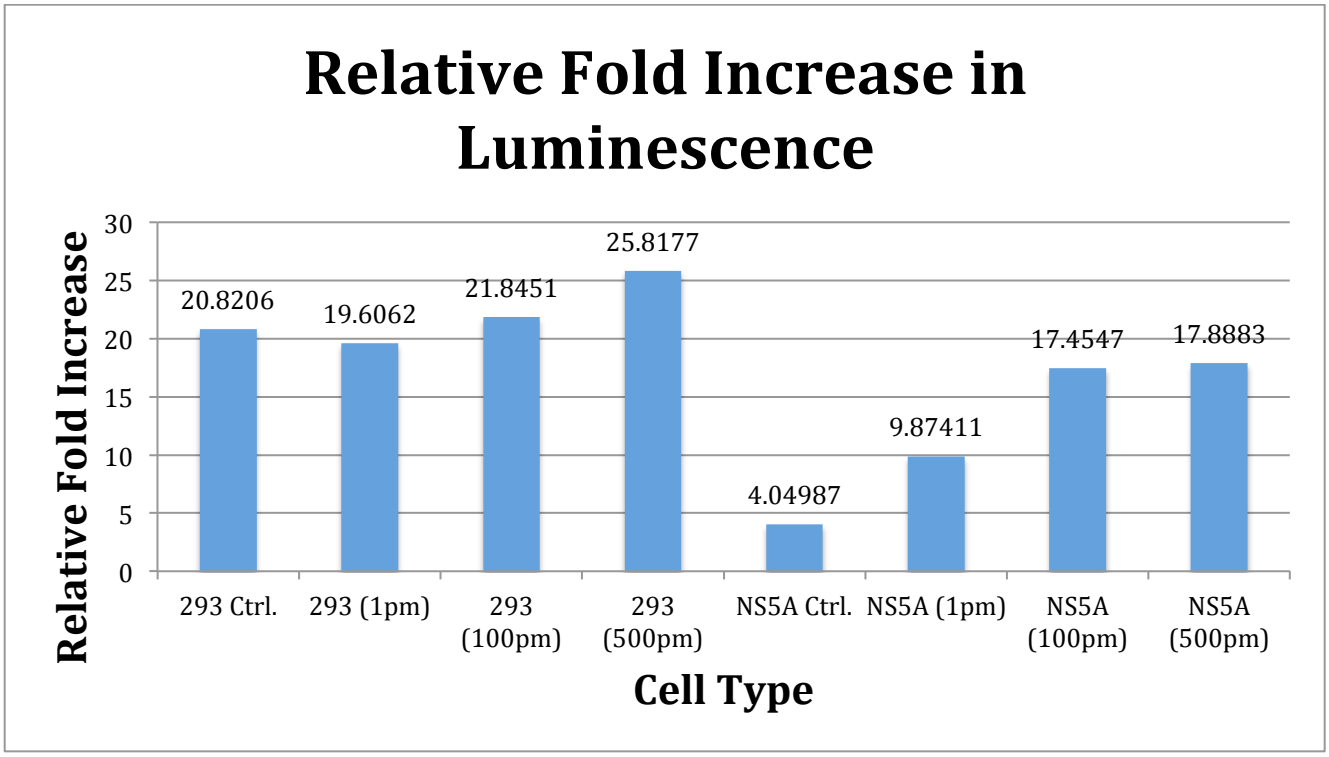

Figure 5: Luciferase Assay comparing 293 HEK control cell line and 293*NS5A-10A treated with varying levels of Daclatasvir.

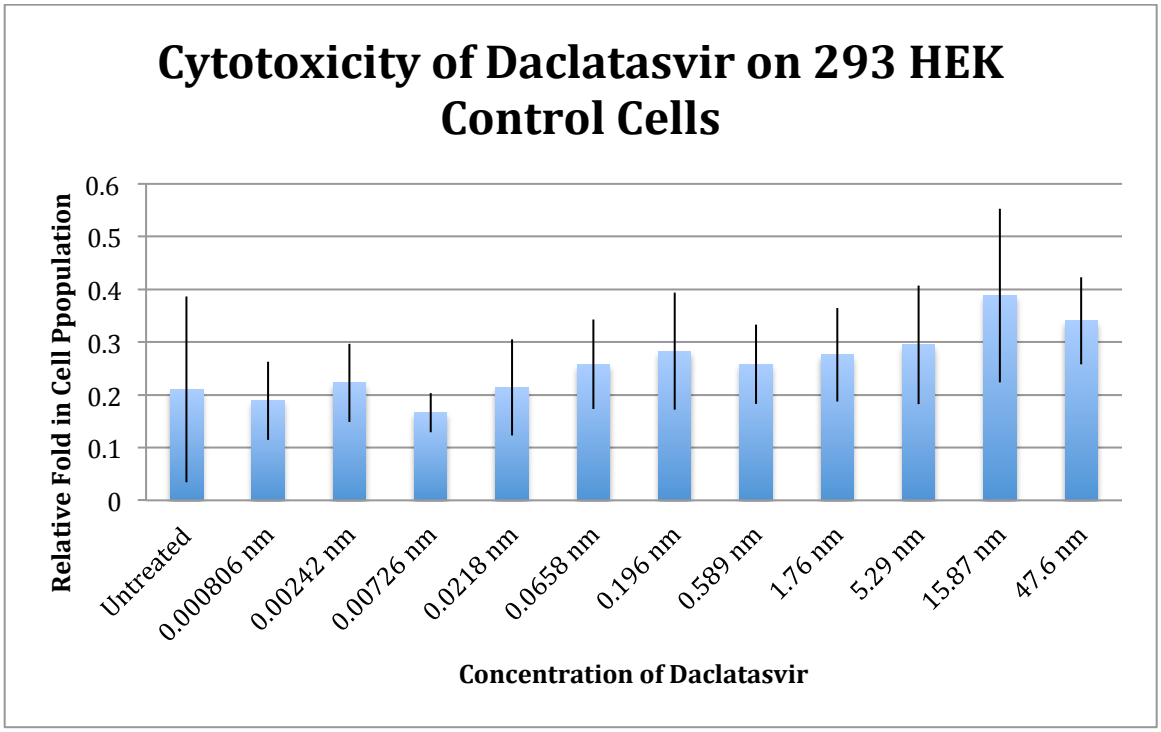

Figure 6a: MTT Assay analyzing the cytotoxic effect of Daclatasvir on 293 HEK Control Cells

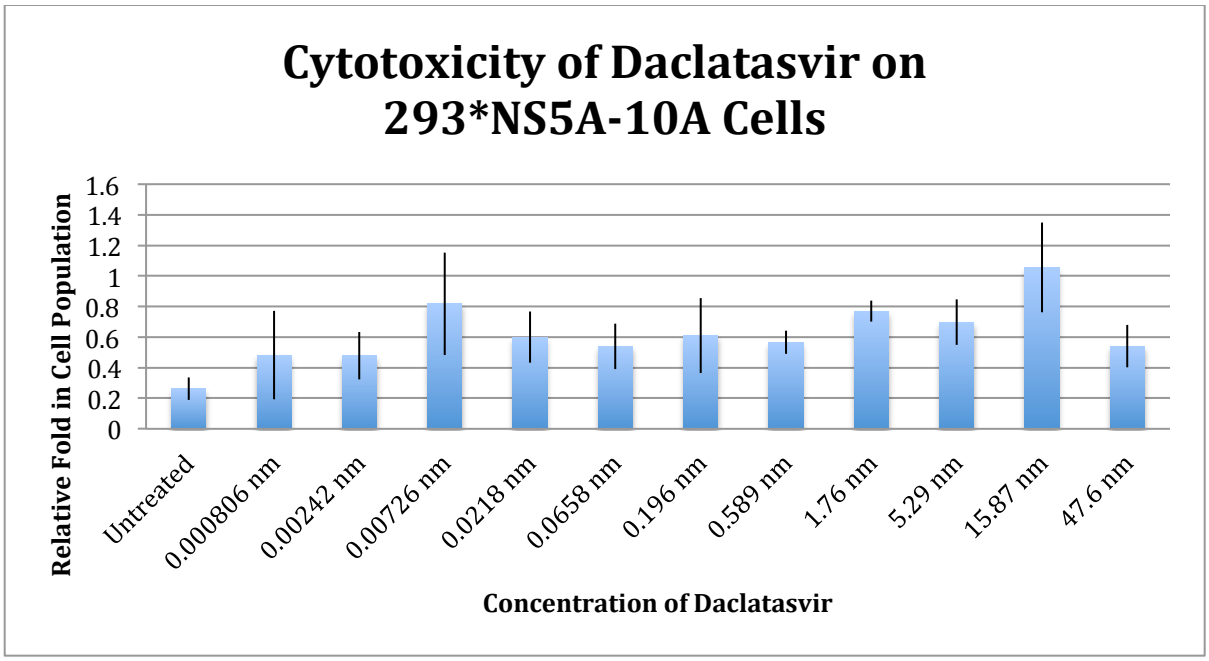

Figure 6b: MTT Assay analyzing the cytotoxic effect of Daclatasvir on 293*NS5A-10A Cells

Discussion

The results obtained from this research project will be used in collaboration with additional studies in the laboratory of Dr. Giridhar Akkaraju to further investigate the replication capabilities of NS5A protein in Hepatitis C Virus. Our research has demonstrated that NS5A-10A renders a block in the antiviral pathway; specifically, this research indicates that NS5A-10A inhibits the process of activating the IFN-β promoter. Furthermore, because we were able to demonstrate that different mutants of NS5A are not able to inhibit this promoter activation, this signifies that the previously demonstrated ability of NS5A-10A to increase viral replication five-fold results directly from its the ability to inhibit IFN-β (Sumpter et. al). Additionally, the ability to inhibit IFN-β promoter activation suggests an explanation for diverse levels of infection. When we inhibited NS5A-10A using Daclatasvir, we witnessed an increase in the cell's ability to activate the IFN-β promoter. The capacity to re-stimulate this pathway indicates shows evidence for further treatment options and potential preventative measures by inhibiting NS5A. Therefore, the results from this research can be used to further understand Hepatitis C infection and develop drugs that can target NS5A, an important protein involved in the pathogenesis of HCV infection.

REFERENCES

CDC. Hepatitis C FAQs for the Public. *Centers for Disease Control and Prevention: CDC 24/7 Saving Lives, Protecting People*. Web. 26 Feb 2014. http://www.cdc.gov/hepatitis/C/cFAQ.htm.

Dimitrova, M., Imbert, I., Kieny, M. P., & Schuster, C. (2003). Protein-Protein Interactions Between Hepatitis C Virus Nonstructural Proteins. *Journal of Virology, 77 (9)*, 5401-5414. doi: 10.11/1128/JVI.77.9.5401-5414.2003.

Feitelson, M.A. (2002). *Hepatitis C Virus: From Laboratory to Clinic*. Cambridge: University of Cambridge Press.

Kwong, A.D., Kim, J.L., Raoi, G., Lipovsek, D., & Raybuck, S.A. (1998). Hepatitis C Virus NS3/4A Protease. *Antiviral Research, 40(2)*, 1-18.

Rhea, S. J., Wange, C., Foy, E., Loo, Y.M., & Gale, M. (2004). Viral Evolution and Interferon Resistance of Hepatitis C Virus RNA Replication in a Cell Culture Model. *Journal of Virology, 78(21)*, 11591-11604. doi: 10.1128/JVI.78.21.11591-11604.2004.

Seth, R. B., Sun, L, & Chen, Z. J. (2006). Antiviral Innate Immunity Pathways. *Cell Research, 16,* 141-147. doi:10.1038/sj.cr.7310019.

Sumpter, R.J., Wang, C., Foy, E., Loo, Y. M., & Gale, M. J. (2004). Viral evolution and interferon resistance of hepatitis C virus RNA replication in a cell culture model. *Journal of Virology, 78(21)*:11591-604.

Von Hahn, T., & Rice, C. M. (2007). Hepatitis C Virus Entry. *Journal of Biological Chemistry, 283*, 3689-3693. doi:10.1074/jbc.R700024200.