Evaluation of antiapoptotic potential of Sulforaphane via NRF2 signaling pathway in a primary culture of PBMCs derived from chronic HCV patients

By

Maheen Rehman

(Registration No: 00000400392)

Department of Biomedicine

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

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Maheen Rehman

(Registration No: 00000400392)

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Department of Biomedicine

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Examination Committee Members

1. Name: Dr Maria Shabbir Signature:

2. Name: Dr Yasmin Badshah Signature:

3. Name: Dr Ishrat Jabeen Signature:

Supervisor's name: Dr Sobia Manzoor

Dr. Sobia Manzoor, PhD
Professor (Tenured)
Dept. of Healthcare Biotechnology, ASAB
National University of Sciences and Technology (NUST), Islamabad

Signature:
Date: 21 Aug - 2024

Date : 23/08/2024

Head of Department

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Abdul Rahman School of Applied Sciences, NUST, Islamabad

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Name of Supervisor Dr Sobia Manzoor

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Signature (HOD): _____

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Dr. Sobia Manzoor, PhD
Professor (Tenured)
Dept. of Healthcare Biotechnology, ASAB
National University of Sciences & Technology (NUST), Islamabad

(Supervisor) Prof Dr. Sobia Manzoor

Biomedicine

ASAB, NUST

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Dedicated To My Parents, Teachers, Sister & Friends

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List of Abbreviations

μg	microgram
μM	micro-Molar
EDTA	Ethylenediaminetetraacetic Acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
IFN-γ	Interferon Gamma
IL	Interleukin
PBMCs	Peripheral Blood Mononuclear Cells
BCL-2	B-cell lymphoma 2
CD-8A	Cluster of Differentiation 8 alpha chain
NRF-2	Nuclear Erythroid factor related factor 2
ARE	Antioxidant response element
CTL	Cytotoxic T lymphocytes
KEAP-1	Kelch like ECH associated protein 1
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
PBS	Phosphate Buffered Saline
SOD-1	Superoxide Dismutase
BIM	Bcl-2-like *protein* 11
MCL-1	Myeloid leukemia cell differentiation protein

ABSTRACT

Various studies show that on average, Hepatitis C virus (HCV) prevalence in Pakistan by percent in adult population is 11.55%. Most of the people diagnosed with HCV infection are aged around 25-35 with ever increasing incidence, around the globe. Our study highlights critical aspects of chronic HCV pathology and its effect on the Immune system. Peripheral Blood Mononuclear cells, specifically CD8+ T cytotoxic lymphocytes and CD4+ T helper cells endorse a hyper-responsive state owing to the levels of viremia and antigenemia caused by chronic Hepatitis C virus. Consequently, these cells become highly dysfunctional. It is well documented that HCV leads to oxidative stress. It also dysregulates antioxidant defense systems. High levels of reactive oxygen species (ROS) lead to oxidation and damage of cellular proteins and organelles, as a result Peripheral Blood Mononuclear cells (PBMCs) become dysfunctional. HCV specific lymphocytes become exhausted due to constant epitope signaling caused due to HCV specific antigens as well as DNA damage caused by high levels of oxidative stress caused in the chronic HCV infection. This leads to the apoptosis of PBMCs via FAS/FAS-L mediated (extrinsic) and intrinsic pathways respectively. Sulforaphane, which is a naturally derived anti-inflammatory and cytoprotective phytochemical can reverse the oxidative stress by activating the NRF2 signaling pathway that can reverse the apoptosis of exhausted virus specific PBMCs. In our study PBMCs were isolated, cultured and treated by optimal doses of Sulforaphane. Cell viability assays were performed to know whether Sulforaphane (SFN) plays a pivotal part in reversing the anti-oxidant stress, cellular exhaustion and apoptosis. The changes at the genetic level are marked by performing RT-PCR, to detect the increased expression of genes involved in anti-oxidant defense systems. The reversal of

these stressors leads to enhanced cell survival, reversal of apoptosis, proliferation and improved functionality required to ward off viral load. Cross validating assays were performed using computational biology tools, including molecular docking and GRIND (advanced alignment independent 3D QSAR) to further validate our findings.

Key words:

Chronic HCV, Viral immunology, Sulforaphane, Apoptosis, Cellular Proliferation.

CHAPTER 1: INTRODUCTION

Hepatitis C virus belongs to the genus Hepaciviral of family Flaviviridae family. It has a positive sense, single stranded RNA genome with a size of 9.6 kb. The RNA genome encodes a polyprotein of 3,000 amino acids. During the HCV life cycle the polyprotein cleaves into four structural (C, E1, E2 and p7) and six nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins (Negro, 2020). HCV appoints cellular proteases to cleave the structural proteins. This results in the self-cleavage of the non-structural proteins. Protease has another function of cleaving interferon signaling molecules and so plays a role in immune suppression (Keikha et al., 2020). Looking at the significance of proteases in promoting HCV it is not surprising that the very first of the DAAs were NS3 protease inhibiters (telaprevir and boceprevir). An important hallmark in the development of HCV treatment was the formation of Sofosbuvir (Ribavirin) which targets viral polymerase. It is not susceptible to drug resistance by different viral variants like other DAAs (Idilman et al., 2021) Irrespective of clinically proven Direct Anti-viral agents (DAA) working against Hepatitis viruses the prevalence of the virus infection continues to be a public health concern. Most of the people diagnosed with HCV/HBV infection are aged around 25-35 with ever increasing incidence, this is a global crisis (Fabrizi et al., 2021). An important cause of it is that monotherapy of with a single DAA can somewhat lead to resistance (Fadnes et al., 2021). There is still no available vaccine for HCV. Along with that economic reasons such as limited access, high cost, unavailability of DAAs, impaired immunity and lack of knowledge about the viral infection as well as progression

of the infection into HCC and relapse make the global eradication of the virus near to impossible soon (Hayes et al., 2022)

Human physiological system can be under oxidative stress, when the amount of ROS production and the removal of oxide is not balanced (Gabriele Pizzino, 2017). ROS are reactive chemical species containing oxygen, for example, hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$). (Zhang et al., 2019). The end result of oxidative stress is cell death. (Pizzino et al., 2017) It is clearly established that HCV leads to oxidative stress, it also dysregulates antioxidant defense systems, leading to chronic hepatitis C and extra hepatic manifestations such as Diabetes Mellitus type 2 (Eslam et al., 2011) cardiovascular disease, lymphoproliferative disorders (Jawad et al., 2021a) and glomerulonephritis (Ozkok & Yildiz, 2014). Biomarkers of oxidative stress are observed in the serum of chronic HCV patients as well as in-vitro systems.(Ayisire & Sayes, n.d.). HCV proteins including core, E1, E2, NS3, NS4B and NS5 trigger oxidative stress. (Jansons et al., 2019). HCV core protein and NS5A have been proven to induce ER stress which leads to the release of cytoplasmic Ca2+ via the inhibition of ER calcium ATPase (SERCA) as well as the leakage of Ca2+ passively (Dionisio et al., 2009). Viral protein NS5A induces the release of Ca2+ which triggers ROS production in mitochondria, leading to activation of NF-κB and STAT-3 (Jawad et al., 2021b). Envelope proteins E1 and/or E2 trigger the stress indicator CHOP protein, cause the accumulation of ROS and activation of NRF2 (Repici et al., 2024). While HCV NS3 can induce ROS production in human Monocytes. (Bureau et al., 2001) Viral pathogenesis is promoted by elevated levels of Reactive Oxygen Species generation. Reports describe increased numbers of ROS in liver tissue samples, lymphocytes and serum obtained from

HCV infected patients. Whereas reduced levels of GSH (Glutathione) are observed in both serum and liver.(Regina Medvedev, 2015). As the AOs (enzymatic or non-enzymatic) get depleted the risk of infection becoming severe increases which manifests as patients getting pre-disposed to Liver fibrosis and Hepatocellular Carcinoma.(Dhiraj Yadav, 2002). Oxidative stress as a potential disease-causing mechanism is indicated by higher concentrations of lipid peroxidation byproducts, such as malondialdehyde (MDA), in the serum and liver of patients with Chronic Hepatitis C (CHC) when compared to healthy individuals (Obiako et al., 2023). Additionally, CHC patients exhibit elevated levels of superoxide dismutase activity and increased biosynthesis of glutathione (GSH). (Boya P, 1999; Larrea E, 1998). Although there is increased biosynthesis of Glutathione, the serum values of GSH remain below the normal cut off value which indicates hyper activated turn-over of the enzyme. (Dhiraj Yadav, 2002).

T lymphocytes endorse a hyper-responsive state partly owing to the level of viremia and antigenemia due to chronic Hepatitis C virus, consequently these cells become highly dysfunctional (Fadnes et al., 2021). Exhausted CD8+ T cells attributed to chronic HCV infection show the following features: 1- Metabolic dysregulation with depolarized mitochondria and ROS accumulation. 2- Upregulation of multiple co-inhibitory signals which promote apoptotic death of T cells. 3- Upregulated histone methyltransferases (HMTs) cause chromatin silencing. 4- Dysregulated Proteostasis, high levels of ROS lead to oxidation and damage of cellular proteins and organelles, this tends to reduce the rates of autophagy. T cells become dysfunctional unable to clear the aggregates of defected proteins and damaged organelles (Barili et al., 2021). This dysfunctional state is termed T cell exhaustion. This leads to the loss of T cell effector functions. This state is achieved by

constant nagging by high levels of viral antigens (McLane et al., 2019). The grade of T cell dysfunctionality varies, it usually starts with IL-2 release, cytolysis and loss of proliferation, this subsequently leads to a more adverse state described by TNF-α and IFN-γ secretion and finally manifests to physical deletion of CD8+ T cells, this is the most terminal stage of exhaustion (Zhang et al., 2023). Some other extrinsic factors also play a part in T cell exhaustion besides persistent epitope signaling including increased activity of T regulatory cells and the loss of CD4+ T cells (Wherry & Kurachi, 2015) Chronic Hepatitis infection causes immune dysfunction and DNA damage in T cell lines because of increased oxidative stress which eventually leads to them exhausting.(Madison Schank, 2021). The results of studies show that cells of Adaptive immunity experience increased mitochondrial load, fewer number of mt-DNA numbers, increased intracellular and mitochondrial ROS production. There is a down-regulated expression of Mitochondrial regulator genes such as peroxisome proliferator-activated receptor 1 alpha (PGC-1α) and mitochondrial transcription factor A (mt-TFA) (Sahin E, 2011; Schank M, 2020). Numerous investigations have documented an expedited process of cellular depletion and senescence in CD8 T cells subsequent to HCV infection, this may play a role in diminishing the likelihood of successfully clearing the virus. (Bengsch B, 2006; Chen DY, 2020). As compared to healthy controls the peripheral mono-nuclear cells of CHC patients show elevated cellular reactive oxygen species (c-ROS), immunoregulatory and exhaustion markers. (Barathan et al., 2015). Apoptotic markers like *TNF, Tp73, Casp14, Tnfrsf11b, Bik* and *Birc8* are highly up-regulated whereas *TNF, Tp73, Casp14, Tnfrsf11b, Bik* and *Birc8* are moderately up-regulated in HCV infected subjects (J. Liu et al., 2022).

Sulforaphane (SFN) is a phytochemical from Brassicaceae family e.g. Broccoli, it is a naturally occurring Isothiocyanate (Dudek et al., 2021). It is said to have anti-oxidant, anti-apoptotic and anti-inflammatory affects in established literature.(Stanciu et al., 2021). SFN is also said to improve cell viability and reduce cytotoxicity. (Sohel et al., 2018). Other than chemoprotection SFN is also involved in redemption of other pathologies such as hyperglycemia, and that of heart, muscle, kidney. Metabolic derangements occur in the patients of chronic hepatitis viral infection patients (Dudek et al., 2021). Sulforaphane is highly chemically reactive due to the electrophile nature of its central Carbon of the Isocyanate (NCS). Cysteine residues are known to be highly reactive with Isocyanates. After entry into the cell Sulforaphane reacts with Kelch-like ECH associated protein 1 (KEAP1) (Adinolfi et al., 2023). This protein rich with cysteine residues is the guardian of many oxidants and electrophiles including isothiocyanates (Kopacz et al., 2020). During normal physiological conditions, KEAP1 targets NRF2 for ubiquitination and proteasomal degradation (Koppula et al., 2022). NRF2 binds to KEAP1 dimer by two distinct motifs (S. Liu et al., 2022a).

CHAPTER 2: LITERATURE REVIEW

2.1 Hepatitis C Virus

2.1.1 History and Origin

The genus of HCV is Hepacivirus. Its family is Flaviviridae. It got the status of a pathogen in 1989 (Negro, 2020). At that time, it was known as non-A and non-B hepatitis. HCV infects chimpanzees and humans. In Pakistan the most common genotype is Genotype 3a. This genotype is present in almost 80% of chronic hepatitis C (CHC) patients (Idilman et al., 2021).

Hepatocytes (liver parenchymal cells) are infected with HCV. HCV is transmitted via contact with infected blood, which may occur with injection drug, transplantation of organ and transfusion of blood (Butt et al., 2020) . It also happens due to exposure to infected blood, through vertical transmission. Lower records are present of transfer of HCV sexually (Lloyd & Franco, 2023) .

2.1.2 HCV Epidemiology

More than 304 million people world-wide are infected with Hepatitis C virus, according to 2022 World Health Organization (WHO) data (Kenfack-Momo et al., 2024) . 1.3 million people died of chronic Hepatitis C virus alone in the year 2022 (Kenfack-Momo et al., 2024) . There is a direct link between chronic HCV and the development if hepatocellular carcinoma (HCC), worldwide. 22% (>100,000) out of 500,000 cases of liver cancer are due to HCV infection. It is reported that 80% of acute cases of hepatitis C, progress to chronic HCV infection (Stasi et al., 2024). Out of the chronic HCV infection cases, 10-20% cases develop various complications like hepatic cirrhosis, just within a period of two to three

decades. 1-5% (Stasi et al., 2024) of the cases develop into liver carcinoma (Kenfack-Momo et al., 2024). This is why HCV is a problem of global importance (Stroffolini & Stroffolini, 2024). There is high prevalence of HCV in India, Pakistan, China and Western Europe, Northern America Australia (Habib et al., 2023). 79% of the Pakistani infected patients of HCV belong to the G3a genotype which causes reduced treatment responses and higher chances of cirrhosis (X. Wang & Wei, 2021a).

2.1.3 Viral Structure and Genome

HCV constitutes of a positively charged single stranded RNA genome. The genome consists of 9600 nucleotides (Triebel et al., 2024). They encode for one polyprotein consisting of 3000 amino acids. Processing of this polyprotein this done via viral and host enzymes to produce following structural proteins: Core (C), enveloped glycoproteins (E1 and E2) and seven proteins which are non-structural namely (P7, NS2/3, NS3, NS4A, NS4B, NS5A and NS5B) (Triebel et al., 2024) . There are six genotypes of HCV majorly (Dearborn & Marcotrigiano, 2020). All differ in terms of their geographical distribution and their response to therapy (Dearborn & Marcotrigiano, 2020). There are variable quasi-species of hepatitis C virus which are all different in terms of the hosts they infect and vulnerability to immune-recognition (Yechezkel et al., 2021).

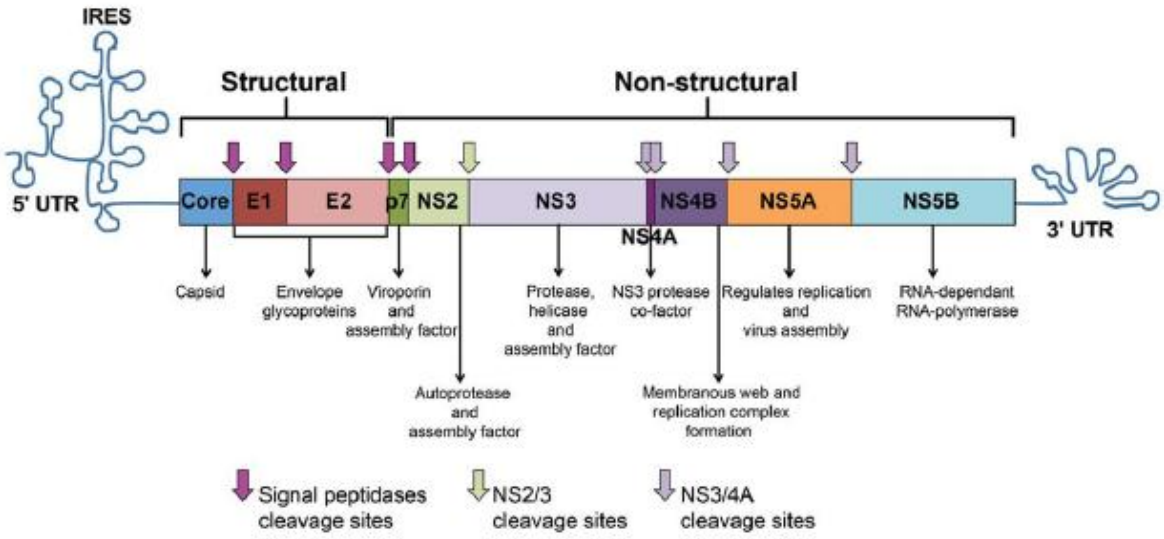

Figure 2. 1 'Genome and polyprotein of HCV'

An open reading frame trapped within untranslated regions (UTRs) of 5’ and 3’ end. The open reading frame translated into a polyprotein which then cleaves into ten HCV proteins, Core protein, E1, E2, p7, NS2/3, NS4, NS5A and finally NS5B.

2.1.4 Treatment response of HCV

Although effective, HCV treatment is only effective if the patients receive it within 1-6 months of infection. Even after receiving the treatment, it is not permanently therapeutic and the patient is always at a chance of developing chronic infection (Stanciu et al., 2021). Previously the treatment option for HCV interferon was only effective for 24 weeks in controlling the viremia (Triebel et al., 2024). Back in the day, standard HCV treatment was PEGylated interferon given in combination with ribavirin. This was generally used as a therapy for HCV infection. Clinical trials for four different types of vaccines constructed for HCV are under clinical trials.

As a result of recent advances in the scope of HCV therapeutics there is an availability of many direct acting anti-viral (DAAs). The following HCV genotypes: 1, 4, 5 and 6 are being currently treated with a combination of Sofosbuvir and Ribavirin which is successful in generating up to 90% productivity towards viral infections and they produce a satisfactory and relatively longer acting virological response (X. Wang & Wei, 2021b). However, the patients with infection from genotype G3a still show less treatment responses (Yechezkel et al., 2021). It is guided to use a combination of SOF + RBV for HCV patients, for a time duration of 48 weeks. However, it is to be noted that most of the research conducted on this treatment are conducted in western countries where other genotypes are more prevalent. For non-cirrhotic patients, it is recommended to use a combination of SOF + RBV irrespective of their relapse or prior treatment is PegIFN + RBV. Other than that, for patients with compensated cirrhosis both SOF/VEL and GLE/PIB are recommended regimens (Zarębska-Michaluk, 2021).

Sofosbuvir, is an RNA dependent RNA polymerase inhibitor and is very effective. Its cons are the treatment cost and the associated viral resistance to this drug (Shabani et al., 2021). There are connected side affects of HCV treatment, including fever, headaches, joint pain. The most major side effect of RBV is hemolytic anemia (Kow et al., 2022). It is estimated that 75% Pakistani population is not able to access the medical facilities. There are many brands of DAAs available in Pakistan but they are not accessible to laymen due cost, lack of medical facilities and ignorance (Shah et al., 2021).

2.2 Viral-Host Interactions

The main targets for HCV infection are hepatocytes. Studies suggest that on average only 10% hepatocytes become infected with HCV. The entry of the virus into the cell happens

through endocytosis mediated by receptor (Chigbu et al., 2019) . The receptors involved in the cellular entry of virus into the cell are CD81, the scavenger receptor (SR-BI), claudin 1 and the LDL receptor (Matthaei et al., 2024) .

After the delivery of virus into the cell, it unravels its coat and its RNA is released into the cytoplasm. The generation of viral proteins is done via the release RNA in the cytoplasm. These proteins along with newly synthesized RNA are packaged to form new virions (Suslov et al., 2022) .

Initially the cells respond by inducing the interferon stimulating genes in the hepatic lobules. This helps in reducing the viral replication up to some extent (Teng et al., 2021) . Irrespective of activation of interferon stimulating genes (ISGs) not much of an anti-viral affect could be noted (Martinez & Franco, 2020) . As a result, a constant persistence in the viremia is seen in the system even up to decades in hepatocytes (Pérez-Vargas et al., 2021) . It is also described that HCV proteins inhibit the JAK-STAT pathway as well as ISGs, the exact way in which they do it needs to be studied in detail (Matthaei et al., 2024) .

2.3 Course of Infection by HCV

2.3.1 Medical and Immunological picture: Acute Infection

After the initial exposure to the virus, appearance of virus in the patients' blood happens as soon as one to three weeks (Antuori et al., 2021). Even after appearance of symptoms, only 50-70 percent of patients develop anti-bodies which can be recognized. After the development of HCV infection serum alanine aminotransferase (ALT) levels start to elevate by eight to twelve weeks (Casey et al., 2019).

This is the time when T cells in a patients' blood start to encounter the virus. One of the characteristic of this virus is delayed immune response, despite high levels of viremia (Y.-R. Li et al., 2020). Symptoms of the infection and high levels of ALT go hand in hand, as a result serum levels of ALT start to reduce as the symptoms start to disappear. Only a small number of patients clear the virus in its acute stage, majority of the patients see a persistent viremia which leads to chronic HCV infection (C.-H. Liu & Kao, 2023). Viral mutants escape the acute phase due to this immunological response. Literature suggests that for true clearance of viral infection, HCV specific CTLs are necessary not just basic neutralizing anti-bodies (Y.-R. Li et al., 2020).

Very early on it was believed that there is an important role of CD4+ T cells in eliminating viral infection (C.-H. Liu & Kao, 2023). Even the resolution of HCV infection was known to be associated with the immunological responses of CD4+ T cells. It was then investigated that immunological response during acute infection by CD4+ T cells is not missing completely (Martinello et al., 2018). The subjects who are unable to generate HCV specific responses are more prone to developing chronic disease. On the other hand, the people who tend to clear the virus from the body show high CD4+ T cell immune responses. The people who cannot clear the virus from the body appear to show low proliferation and cytokine production. It is also studied that for regular generation and response of CD8+ T cells signified health of CD4+ T cells in important (Matthaei et al., 2024) .

2.3.2 Medical and Immunological Picture: Chronic Infection

By chronic infection it means that there are detectable levels of RNA of HCV in the blood of an individual post six months of infection. Epidemiological studies suggest that more

than 85% of people infected with HCV develop chronicity (D'Ambrosio et al., 2021). Clinically the picture can be seen as cirrhosis, end stage liver disease and cancer. A hallmark of chronic HCV infection is elevated losses of HCV specific CD4+ T cells (Fabrizi et al., 2021). There are many ways by which HCV viruses generate mutations in their MHC class 2 specific epitopes which leads to reduced CD4+ T cell response (Lampertico et al., 2020). New literature suggests that viruses producing escape mutations which can totally hide from are very uncommon and they don't play an essential impact in chronicity of infection (Lobo et al., 2022) .

Not only CD4+ T cells as well as CD8+ T cells are also disrupted functionally in chronic HCV infection (D'Ambrosio et al., 2021). As a result, it is impossible to control viral infections based on natural host immunity (Marascio et al., 2022). The dysfunctionality of CD8+ T cells is also based on the reason of antigen escape mutations. The functional exhaustion of these cells is due to viral persistence which in turn leads to poor clearance of virus in chronically infected patients (Page et al., 2021).

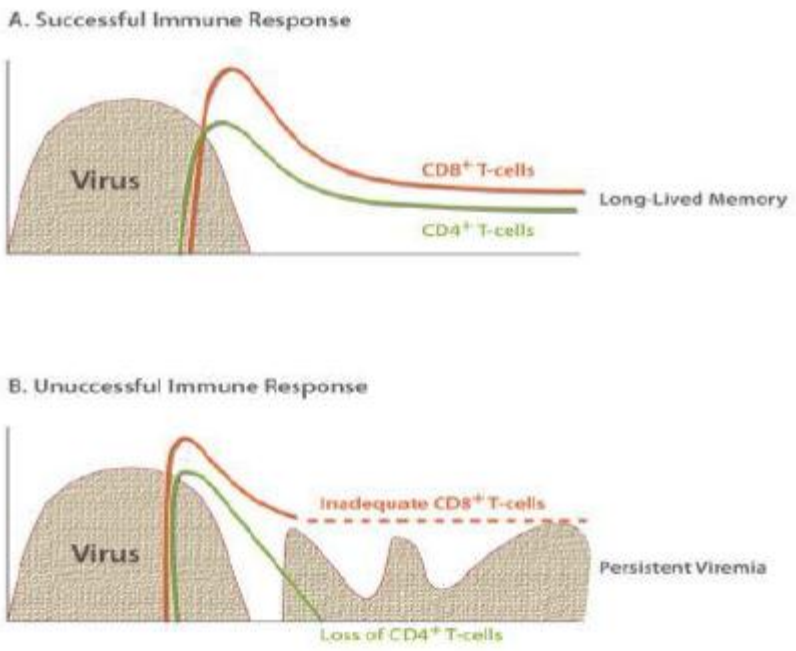

Figure 2. 2 'Graphs representing the response of Immune system to viremia.'

2.4 T cell exhaustion related to Chronic Viremia

Irrespective of the T cell responses and presence of neutralizing anti-bodies there are enough signs of persistence in the host micro-environment (Deguit et al., 2019). To help the body to develop an adaptive immune response against viral infections, there is a proposition of several intervening mechanisms (J. Z. Li et al., 2020). The main issues being evasion of the virus, emergence of quasi-species, cell to cell contact, and interactive sessions between glycoproteins of the virus and high-density proteins of the body. Viral escape mutants cause T cell failure and T cell exhaustion (Papavassiliou & Musti, 2020).

While a person is infected with chronic viral infection, 0.05-2% of the circulating CD8+ T cells are HCV specific CD8 T cells (Scharf et al., 2021). In reference to the HCV infection, these CD8 T cells have been most studied. It is said that in comparison to normal CD8 T

cells, HCV specific CD8 T cells produce less granzymes, perforins, give limited CTL function and even a reduced ability to produce IFN-gamma, TNF-alpha. The HCV specific CD8-T cells are called as exhausted T cells. Whenever these exhausted cells are stimulated by a cognate antigen, CD8 T cells produce less IFN- gamma and even less perforin (Zhang et al., 2023).

It has been confirmed that HCV specific CD8+ T cells are more affected in terms of exhaustion and failure (D'Ambrosio et al., 2021). This exhaustion is marked by high levels of PD1, LAG3, and TIM3. It commutatively accepted that increased in inhibition markers has high association with lymphocytic apoptosis (Scharf et al., 2021). In the same manner, the CD8+ T cells which are present inside the liver show even higher levels of PD-1 and are more associated with apoptosis (Beura et al., 2022) . Not one receptor is involved in exhaustion, it is however a combined effect many separate inhibitors and this effect can be reversed by cutting or knocking down these receptor (Raju et al., 2021).

HCV also stimulates the pro-apoptotic pathways to cause an induction in T cell death. As a result, the immune system continues getting weaker and weaker (Correa-Rocha et al., 2018). Upregulation is seen in pro-apoptotic members: Bcl-2 (BIM). On the other hand, downregulation of a differentiating protein (MCL-1). There needs to be a balance kept between BIM and MCL-1 (Deguit et al., 2019).

2.5 Apoptosis

Apoptosis is death of a cell in a programmed order. Its main reason is to maintain cellular homeostasis, morphology and defense in quite many organisms (Bertheloot et al., 2021). All apoptosed cells die without causing damage to the adhering milieu. It is a completely

interconnected procedure which always signals to activate essential caspases and other pro-apoptotic proteins (D'Arcy, 2019) . This causes a shrink in the size of nucleus and the blebbing of cellular membranes. Apoptotic bodies produced are ingested by phagocytic cells. In this way it is made sure that cellular content is not spread around the micro-environment (Kashyap et al., 2021).

For regulating apoptosis there are two major mechanisms, one is intrinsic pathway and the other is extrinsic pathway. If there is binding of death receptors such as FAS and Tumor necrosis factor to their specific ligands which paves a path for the continuation of extrinsic mechanism of apoptosis (Ketelut-Carneiro & Fitzgerald, 2022). On the other hand, intrinsic pathway also known as (mitochondrial pathway), means when non receptor mediated signaling takes place inside the cell which itself triggers mitochondrial to activate apoptosis (Majtnerová & Roušar, 2018). For death, mitochondria need to permeabilize their membrane which is made possible by BCL-2 and family (Obeng, 2021). Overall, for the mechanistic signaling of apoptosis, a family of cysteine proteases and caspases is initiated. All these molecules are intrinsically synthesized inside the cell. They become active after cleaving. Activated caspases can activate other proteins along the line (Samir et al., 2020).

2.5.1 Intrinsic Pathway of Apoptosis

A receptor independent mitochondrial pathway. This is mediated by intracellular signaling, from within the cell internally (Sorice, 2022). DNA damage, stress, loss, or other factors which affect cell survival. This pathway is regulated by a balance between anti-apoptotic and pro-apoptotic signaling which is initiated from inside the cells (Xu et al., 2019). The pathway can be moved into two ways, post membrane permeabilization: 1) Apoptosis protease activating factor 1 (APAF-1) dependent pathway and 2) Pathway independent of

APAF-1. The first route is dependent on activation of pro-apoptotic BCL-2 family members which is followed by discharge of cytochrome c through the mitochrondias and this is followed by formation of apoptosomes. Activation of pro-caspase 9 is produced by apoptosomes. (Zheng & Kanneganti, 2020) This is then followed by release of a cascade of caspases (Ketelut-Carneiro & Fitzgerald, 2022). On the contrary, APAF-1 which is not dependent on APAF-1, but it is dependent on release of DIABLO proteins from the release of permeable membranes which again causes activation of effector caspases and leads to the initiation of caspase cascade (Kashyap et al., 2021).

The subfamilies of the Bcl-2 proteins are: 1) pro-apoptotic members Bak and Bax. 2) anti-apoptotic members Bcl-2, Bcl-xl and Bcl-w and A1. And 3) pro-apoptoti BIM (Devarajan, 2023).

BIM (BH3 only protein) can initiate apoptosis by both oligo-merization of Bax and Bak (Kumar & Chauhan, 2021). It may also do it indirectly because it has the tendency to soothen and neutralize anti-apoptotic members of Bcl-2 family (Loren et al., 2022).

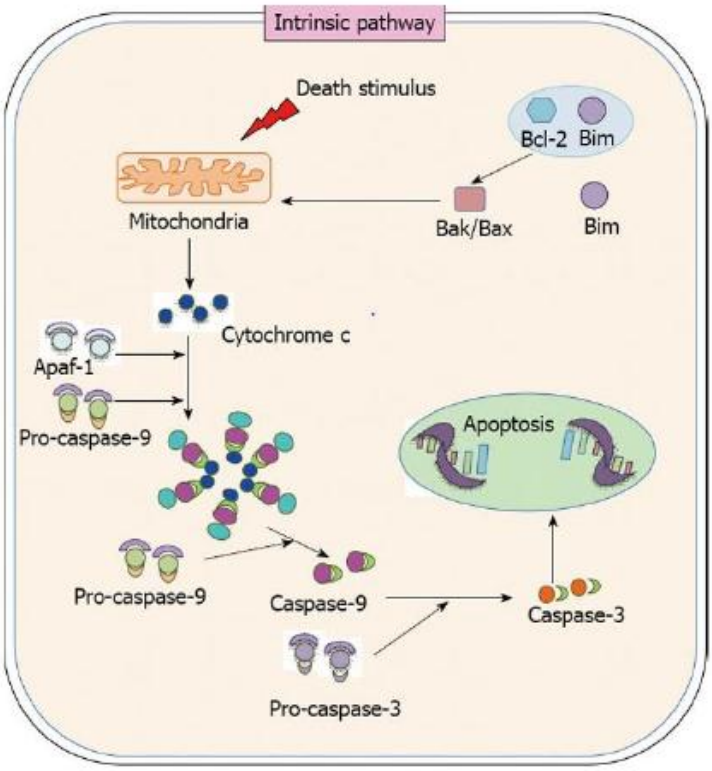

Figure 2. 3 'Intrinsic pathway associated mitochondrial apoptosis.'

The activated BIM favors the upregulation of Bax and Bak this causes the soluble permeabilization of mitochondrial membranes and they release cytochrome c. Apoptosomic structures are created by condensation of Procaspase 9, APAF-1 and cytochrome . This apoptosome activates caspase 9. Caspase 3 is activated because of caspase 9. This step of the intrinsic pathways triggers apoptosis.

2.5.2 BCL-2 interaction mediator of cellular death (BIM)

Known as the molecule of suicide, BIM is a member of Bcl-2 family belonging to BH3 class (Al-Zubaidy et al., 2022) . This suicidal molecules allow the cell to pass out peacefully. After sequestering anti-apoptotic proteins of the family of Bcl-2 like Bcl-xl, Bcl-2 and Mcl it is said to nullify their effect (Amidfar et al., 2019). BIM is known to have three important isoforms, extra-long, long and short. The involvement of the short isoform is the most relevant in apoptotic literature (Amidfar et al., 2019, p. 2).

Literature reports upregulated levels of BIM owing to the stress on the endoplasmic reticulum caused by chronic viral infections. FOX and CEPB transcriptional factor are involved in regulating the transcriptional levels (da Silva Lawisch et al., 2022). On the other hand, proper immune responses by CD8+ T cells are promoted by TNFR associated transcriptional factor also called as TRAF1 which has a negative and antagonistic relation with BIM (Masoud et al., 2022). After treating the CD8+ T cells with ionmycin, taxol and y irradiation lymphocytes and thymocytes are reported to be resistive towards apoptosis because they downregulate BIM which is highly associated to apoptotic induction (Moradipour et al., 2022) .

Already previously discussed that counteraction of HCV is done by active CD8+ T cell responses (Raj S et al., 2023). However, because of antigenic stimulation most of CD8+ T cells start to delete via signaling of BIM and the other are impaired in terms of functionality. In chronic viral infection mediated T cell apoptosis dysregulated and low expression of anti-apoptotic members Mcl and Bcl -2 which block the activity of BIM (Vardiyan et al., 2020).

2.5.4 MCL-1

Myeloid leukemia cell 1 (MCL1) is a protein which is transcribed by an early gene which can become activational by differentiation of myeloid cells (Al-Odat et al., 2021). This is contributive towards cellular viability and proliferations. As the Ca2 + ions are disallowed to enter the mitochondrion by MCL-1 apoptotis is stopped from happening (Bolomsky et al., 2020; Denis et al., 2020). There are two known variants or transcripts of MCL-1 in terms of size long, short and long is associated with cell survival and inhibits apoptosis (Fletcher, 2019). Short one of the other hand promotes apoptosis (H. Wang et al., 2021). Rapid loss of these T and B cells can be studied by knocking down MCL-1 longer transcript. For the proper development and growth stages to be accomplished by lymphoid cells, MCL-1 sabotages BIM (Pervushin et al., 2020).

2.6 Sulforaphane

Sulforphane [1-isothiocyanate-(4R)-(methylsulfinyl)butane] which is naturally occurring isothiocyanate produced naturally by cruciferous vegetables such as broccoli (D. Li et al., 2021). It is dietary in nature and is developed by action of myrosinase enzyme action on glucopharnin enzymes (Mohamadi et al., 2023).Vegetables such as Brassica broccoli, brussel sprout and cabbages contain sulforaphane. It is proven to have cytoprotective and non-cancerous nature that's why scientists are doing recent advances on this topic (Al-Odat et al., 2021).

It is not a directly acting anti-oxidant class. But it is believed that in can create an upregulation in cytoprotective proteins and the enzymes of anti-oxidant response elements genes (Samir et al., 2020). This is done via NRF2-ARE signaling pathway. Some examples of anti-oxidants on which the effect of sulforaphane is studied widely are, hem oxygenase

1, glutathione S and glutathione reductase. To be precise sulforaphane is groundbreaking both ex-vivo and in-vivo to reduce oxidative stress, damage of tissue n cells (Otoo & Allen, 2023) .

2.6.1 Cytoprotective effects of Sulforaphane

sulforaphane is recorded to have set up a phase two detoxification set up. Where it activates NRF2 (Qi et al., 2021; Schepici et al., 2020; Yagishita et al., 2019). Which is a modulator of transcription and activates the transcription of ARE genes which are involved in anti-oxidative responsive elements. There are several clinical trials as well, which include proper ingestion of Sulforaphane as it is an extract obtained from green vegetables (Schepici et al., 2020). In a dose of 200 mg of one day. In this way the oxidative stress can be reduced. Not only this, the anti-hypertensive effect of sulforaphane has also been studied which it performs by balancing the activities of glutathione and glutathione reductase (Mohamadi et al., 2023). This balance of affect is seen in heart, kidney, aorta, interstitial and tubules. These beneficial effects are noticed because of suppression of NFKB pathway by sulforaphane both independently and directly (Treasure et al., 2023).

2.7 Regulation of KEAP/ARE AND NRF2 PATHWAY

The biggest regulator of cyto-protective responses are Keap1-ARE and NRF2 pathway (S. Liu et al., 2022a). This calculates the sensitivity of the cells to measure and manage the oxidative stressors in such a way that they induce the detoxification of anti-oxidant enzymes (Kopacz et al., 2020; Koppula et al., 2022). The pathways is comprised of three components which are Kelch associated ECH proteins (Baird & Yamamoto, 2020), nuclear erythroid related factor and responsers of anti-oxidants genes (ARE). The pathway is shown in figure (Bellezza et al., 2018; S. Liu et al., 2022b; Ulasov et al., 2022).

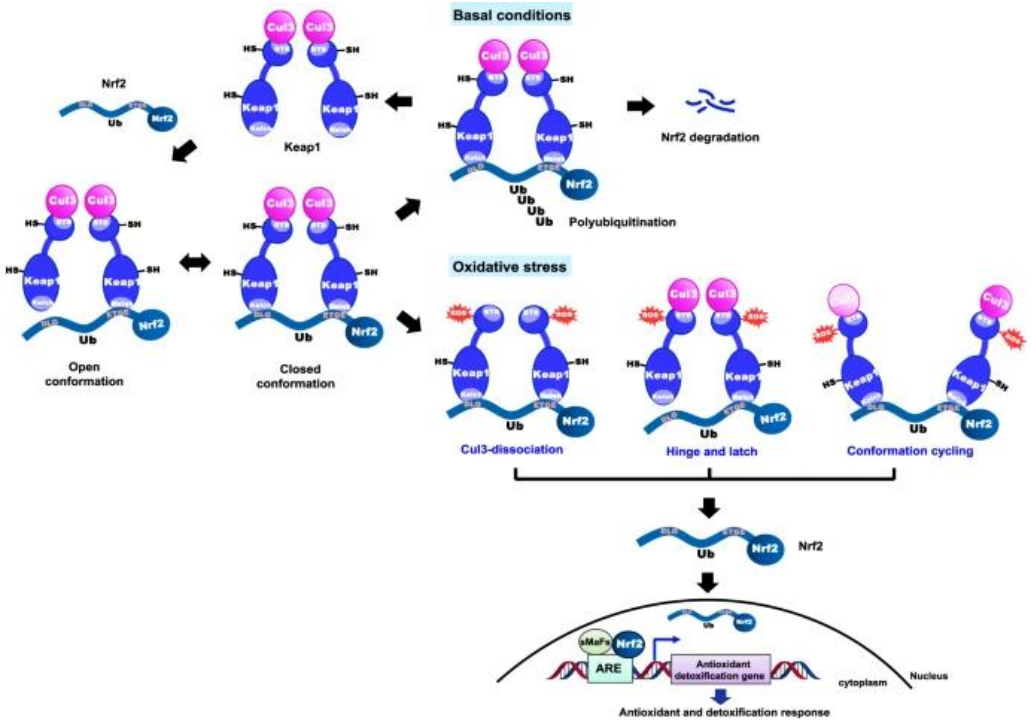

Figure 2. 4 'KEAP-1 NRF2/ARE signaling pathway'

Ubiquitinilation of NRF2 is promoted becaused of Cul-3 which is a ubiquitinylation proteins attached to the BTB domain of KEAP-1. This causes the sequestration of NRF-2 in a cyclic manner and disallows the transcription factor to translocate to the nucleus. As a result the ARE genes don't get activated to transcribe anti-oxidant genes.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Participants

Blood samples were collected from Institute of biomedical and genetic engineering center (IBGE) in Dr. A. Q. Khan Research Laboratories (KRL) hospital, Islamabad. All blood samples belonged to patients having chronic HCV. Approval from Director IBGE was taken prior to sample collection. Candidates ranging from 18- 50 years of age were enrolled in the study. Both written and verbal consent was taken from all subjects who volunteered for the study. 8 ml of peripheral blood withdrawal was made by a trained phlebotomist in EDTA coated purple top vials. It was made sure that samples get processed within 8 hours of blood collection.

3.2 Patient selection criteria

3.2.1 Inclusion criteria

The patients who were relapsers' or non-responders to standard HCV treatment (Ribavirin + PEGylated interferon alpha) with detectable levels of viral RNA in blood up to 6 months of completion of therapy.

3.2.2 Exclusion criteria

The patients who were infected with other viral infections such as HBV or HIV as well as patients who had co-morbidities like diabetes or chronic renal disease were excluded from the study. Patients with in-complete data and those actively under treatment were also ruled out from this study.

3.3 Extraction of PBMCs from Blood

Peripheral Blood Mononuclear Cells were isolated using Lymphocyte Separation Medium (LSM) purchased by MP Biomedicals™ according to the user's manual from 8 ml of whole blood. LSM separates different components of blood according to their density based on the protocol of Ficoll density gradient centrifugation in 15 ml Falcon tubes. Blood samples get fractionated into distinct visible layers after centrifugation. Erythrocytes and granulocytes settle at the bottom. Above this layer is Histopaque and on top of it is the plasma containing platelets. Right between the layers of Histopaque and Plasma is the buffy coat. The buffy coat at the interface has lymphocytes, which are collected. Isolated PBMCs were then mixed with 1x RBC lysis buffer and left for incubation for ten minutes. This is to make sure that any RBC contamination is eliminated. The suspension was centrifuged at 300 x grams at room temperature for ten minutes and then washed with PBS twice. The PBMCs were then cultured in for 4 hours at 37°C with 5% CO2 supply, in phenolic RPMI 1640 medium.

3.4 Trypan Blue Exclusion Assay and Cell counting

One of the most trustable stains for the detection of viable cells via cell counting is Trypan Blue. The mechanism of its action is Exclusion, as it distinguishes live cells from dead cells. In principle this assay states that viable/live cells pick up the dye as a result they remain translucent while the dead cells (non-viable) cells don't take up the day and will be stained as blue. These difference can be seen during the visualization of cell morphology under the microscope.

According to the protocol, 10ul of cells suspended in RPMI-1640 media and 10ul of Trypan blue dye were taken in an Eppendorf in equal 1:1 ratio. The concoction was allowed to rest for 10 minutes so that the cells had the time to take up the dye. The cover slip was put in place on a Hemocytometer and 10ul of suspension mixture was dispensed in one of the chambers of Hemocytometer. First the live cells were counted in the 1mm central chamber of Hemocytometer, then cells were also counted four other 1 mm corner squares. Non-viable cells appeared blue. Cell count was determined after visualization of cell morphology under the microscope at 20X magnification power.

Cells per mL= the average count per square x dilution factor x 10^4

Total cells = cells per ml x the original volume of sample

Cell viability (%) = total viable cells (unstained cells) + total cells (stained + unstained) /100

3.5 MTT Cell Viability Assay

Study of (Gonçalves et al., 2015) was followed, to seed previously cultured human PBMCs in 96 well plates, in a quantity of 5 x 10^4 cells per well. Cells were seeded in triplicates for both healthy (control + treated with L-SFN) and Chronic HCV+ PBMCs (untreated + treated with L-SFN). It was made sure that the total volume of cell suspension in each well is 100 µl. Both plates were wrapped in aluminum foil and were incubated for 24hrs and 48hrs at 37°C with 5% CO2 supply.

After completion of incubation for the respective plates, 10 µl MTT (tetrazolium) dye solution (5mg/ml) by Sigma Aldrich was prepared in PBS while maintaining its (pH at 7.4). Freshly prepared MTT dye was dispensed in each well, and further incubated for 4 hours at 37°C with 5% CO2 supply. After the incubation with the dye completed 100 µl DMSO was added to each well to solubilize the formazan crystals. The plates were then further incubated overnight at 37°C with 5% CO2 supply. Microplate (ELISA) reader by Bio RAD was used to assess the absorbances of the solution at a wavelength of 550 nM. The cell viability in percentage was calculated for both control and test samples by using the following formula:

AC and AT are the means of the absorbances of treated and untreated (control) cells. AB on the other hand is the means of the absorbance value of wells without the cells, i.e. the blanks.

3.6 Simulation of PBMCs with L-SFN In-vitro

Peripheral Blood Mononuclear Cells were extracted from whole blood and resuspended in phenolic RPMI 1640 media (Sigma Aldrich™) supplied with 5% Fetal Bovine Serum (FBS) (Sigma Aldrich™) and 1% penicillin and streptomycin (Pen-Strep Sigma Aldrich™). To adjust the pH of the media, Sodium Bi carbonate was added. In a quantity of 4x10^6 cells/2ml/well cells were seeded in 6 well plates and were simulated with L-SFN at concentrations of 20 µM. In parallel well untreated cells were used as controls. Cells were then allowed to culture over-night at 37 ◌C with 5% CO2.

3.7 Time and Dosage Optimization of L-SFN Simulation

For optimization of time and dosage of L-SFN, PBMCs underwent MTT cell viability assay at five different concentrations, 10µM, 20µM, 30µM, 40µM and 50µM at the time points of 24 hours and 48 hours. Optimal dose and time point was chosen on the qualitative and quantitative basis of absorbance values and cell viability of PBMCs under treatment.

3.8 Percent Cell Viability Assessment

Percent cell viability of the untreated and treated PBMCs of both healthy HCV infected cultures was calculated by the following formula

$$\text{Viability \%} = ((AT - AB) / (AC - AB)) \times 100$$

3.9 Extraction of Cellular RNA

Cells were harvested from the wells of 6 well plates, after the duration of optimal dose was completed. Harvested PBMCs were washed with PBS, then mixed and homogenized thoroughly in 1000 µL TRIzol™ reagent by Invitrogen™. The microcentrifuge tubes were incubated at ice for five minutes. For the enhancement of cell lysis glacial acetic acid (GAA) was used. The sample was again kept under the incubation of ice for ten minutes. Next, 200 µL of 100% chloroform was added and the sample was vigorously shaken in a vortex for 15 seconds. For phase separation this step is very essential. After that centrifugation was performed at 12000 x rcf for 20 minutes at 4˚C. This step test is isolated into three layers; upper fluid layer containing RNA, white hued interface containing DNA and red phenol-chloroform layer at the base involving proteins and proteinaceous debris. The aqueous layer containing RNA is painstakingly suctioned with micropipette and moved into new micro-centrifuge tube. 500uL of chilled isopropanol is then included in the

aliquot and left for 10 min at ice. RNA was precipitated after centrifugation at 12000 x rcf for 20 minutes at 4˚C.

After discarding the supernatant, the RNA sample was washed twice with 1mL of 75% ethanol (chilled). The sample was centrifuged at 7000 x g for ten minutes and RNA pellet was air dried in laminar flow hood for thirty minutes. The total RNA was stored at -20°C for further use.

3.10 Quantification of RNA

Nanodrop was used for the detection of quantity and quality of extracted RNA. Concentration of RNA is determined at a wavelength of 260 nm while the quality of the product being analyzed is checked at a ratio of 260/280 nm. 1 µL of RNA sample previously suspended in Nuclease free water was for the assessment of RNA quantification. Pure nuclease free water was used a blank to ensure the transparency of the procedure.

3.11 cDNA synthesis

Extracted RNA was used to synthesize cDNA by following the users' manual of Solis BioDyne™ cDNA synthesis kit. Oligo (dT) primer binds specific specifically to the polyA tail of mRNA. As a result, RNA gets converted into cDNA. For the processing of cDNA synthesis there are two major steps. For the first step following components needs to be added into the reaction mixture:

Step 1

RNA sample	1µg
Oligo (dT) primer	1µL
Nuclease-free water	up to 12µL

All these components were added in a PCR tube, mixed and incubated at 70°C for 5 min and then quickly chilled at ice for next 5 minutes. For the second step these components need to be added in the same PCR tube.

Step 2

Mixture of Step 1	12µL
5 x Reaction Buffer	4µL
Ribolock (RNase Inhibitor)	1µL
dNTP Mix (10mM)	2µL
Revertaid-Reverse Transcriptase	1µL
Total Quantity	20µL

For the optimized results the amplification conditions used were at 95°C for 5 minutes for initial denaturation. The temperature profile used was denaturation at 95°C for 45 seconds, annealing at 55- 60°C for 45 seconds and extension at 72°C for 45 seconds, final extension was carried out for 10 minutes at 72°C. Amplification was carried out for 35 cycles.

The cDNA template formed post amplification was mixed with 6 x loading dye before running on 2% agarose gel stained with ethidium bromide. 50 bp DNA ladder (GeneRuler™ ready-to-use by Fermentas) to visualize using Dolphin-Doc™ UV trans-illuminator. Sharp band of required product size is a pet sign of successful amplification.

3.12 Primer designing

Primers for human NRF2 (NFE2L2), Superoxide dismutase (SOD1) and CD8A were obtained from Invitrogen™. The sequences of all the primers were retrieved from the OriGene website. Their catalogue numbers are (NM_006164), (NM_000454) and (NM_001768). The primer used are:

Genes	Template DNA Primer	
NRF2 (NFE2L2) (Macrogen, Inc)	Forward Sequence	5’ CACATCCAGTCAGAAACCAGTGG 3’
	Reverse Sequence	3’ GGAATGTCTGCGCCAAAAGCTG 5’
Superoxide Dismutase (SOD1) (Macrogen, Inc)	Forward Sequence	5’ CTCACTCTCAGGAGACCATTGC 3’
	Reverse	3’ CCACAAGCCAAACGACTTCCAG 5’

	Sequence	
CD8A (Macrogen, Inc)	Forward Sequence	5’ ACTTGTGGGGTCCTTCTCCTGT 3’
	Reverse Sequence	3’ TGTCTCCCGATTTGACCACAGG 5’

Table 3. 1 'Sequence of Forward and reverse primers.'

3.13 Optimization of Amplification Conditions on Real Time PCR

The amplification conditions were tested on Applied Biosystems 7300 Real-Time PCR System after extracting 1 µg of total RNA from PBMCs culture, which was then converted to c-DNA using SOLIScript® RT cDNA synthesis kit. The gene expression of NRF2 (NFE2L2), SOD1 and CD8A was normalized with a housekeeping gene i.e. Glyceraldehyde 3- phosphate dehydrogenase GAPDH, by proceeding with the real-time polymerase chain reaction.

Forward and reverse primers for the house-keeping gene GAPDH 10µM were acquired by Thermo Scientific™. The reaction mixture for real-time PCR had the following recipe:

5x Hot FIREPol® EvaGreen® qPCR mix plus ROX by Solis BioDyne	5 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
c-DNA Template	5µL

Water, Nuclease Free	Up to 20µL
Total volume	20µL

3.14 Relative quantification of primers with GAPDH

The m-RNA expression of NRF2, SOD1 and CD8A was normalized with a reference gene, GAPDH by using the following formula:

$$\frac{2\ \Delta Ct\ (Test) = 2\ (Ct\ (NRF2/SOD1/CD8A) - Ct\ (GAPDH))\ Test}{2\ (Ct\ (NRF2/SOD1/CD8A) - Ct\ (GAPDH))\ Control}$$

3.15 Molecular Docking

13.15.1 Retrieval of structures for Molecular Docking

A library of 30 compounds including R-SFN which are inhibitors of KEAP1 were retrieved from PubChem. The PubChem ID of sulforaphane is (CID: 5350). The co-crystallized PDB structure of NRF2-Keap1 protein complex was retrieved from Protein Data Bank PDB with an (ID: 2FLU) from rcsb.pdb.org.

3.15.2 Prediction of Bioactivity and Drug Likeliness

Assessment of performance ability of drug is on the basis physico-chemical and biological properties. Lipinski's Rule of five was the test criteria to determine the drug likeliness of the compounds in the formed library. This rule states that the molecular weight of compounds should be <500, log p <5 and number of hydrogen bond acceptors <10. The number of hydrogen bond donors should be <5. The prediction of bioactivity score and drug likeliness was done using SwissADME(swissadme.ch/index.php).

3.15.3 Molecular Docking and Visualization

Docking was done via Molecular Operating Environmental-Docking (MOE) 2015. The co-crystallized NRF2 was removed from protein and only chain A was kept. The structure of Protein was prepared by energy minimization through the Amber99 force field of MOE. Then, the test compounds underwent energy minimization. After energy minimization ligands were provided with polar hydrogens to adjust their potential energy. The oriented ligands were then saved as a new data base in the format of MDP. A forcefield of MMFF94x was applied. Other than that, bind site was chosen and dummies were established over the helix.

After this the process of docking was started, different times were utilized to produce 10 poses for each ligand. The scoring function used to optimize the poses was the London dG-scoring function which was adjusted twice y triangle Matcher. Surface mapping, validity patterns and interaction analysis were exported into the database during the docking process. The docking results were visualized using PyMol.

3.16 GRIND (Grid Independent Molecular Descriptors Analysis)

5 best 3D molecular confirmations per ligand were obtained from clusters containing docked ligands, along with their inhibitory potencies (pIC_{50}). All this data was imported in pentacle software version 1.06 to construct a grind model. Different probes were used to calculate molecular interaction fields (MIFs). The probes were namely N1, O, DRY, and TIP. N1 refers to amide N which represents hydrogen bond donor, O probes which refers to carbonyl O and represents hydrogen bond acceptor. DRY is indicative of hydrophobic

region and TIP refers to hotspots of the virtual site. Probes were placed with the help of a grid and total energy of interaction was calculated at each node.

An algorithm named AMANDA was used to select the most relevant and highly significant MIFs. It also evaluated structural characteristics of the dataset used to generate GRIND descriptors. After that encoding was performed which used CLACC algorithm. This algorithm chooses consistent nodes by adjustment of compounds according to their moments of inertia. The final grind model was chosen with PLS (partial least square) analysis using LOO (leave one out) method. It was made sure that the method chosen has statistically significant R^2, q^2

CHAPTER 4: RESULTS

4.1 Clinical Data of Collected Samples

Samples of peripheral blood belonging to chronic Hepatitis C virus were retrieved from KRL hospital, Islamabad. It was made sure that only samples from relapse or non-responder category were collected. It was also made sure that the patients who volunteered in the study didn't have any other viral infections such as, Hepatitis B, HIV or co-morbidities such as Diabetes or chronic renal disease. Samples also got screened for other infections. The clinic-pathological data of the patients is given in the following table:

Table 4. 1 Clinical and chemical data of patients.

Sr No	**Age**	**Gender**	**ALT** (4-40U/L)	**ALP** (35-107U/L)	Viral Titer (IU/mL)
1	22	F	71	107	4248147
2	31	M	241	532	7.8×10^6
3	19	M	87	191	2.5×10^5
4	42	F	101	457	5.9×10^4
5	27	M	107	426	2926677
6	49	F	75	219	2×10^4
7	32	F	89	199	124356
8	41	M	91	328	3.8×10^6
9	29	M	65	143	2269998

4.2 Cell Culture

4.2.1 PBMCs Isolation from Peripheral Blood Samples

4 mL of uncoagulated blood was used to extract Peripheral Mononucler Blood Cells by density gradient centrifugation with the help of Lymphocyte Separation Medium. The buffy coat in white mesh color was retrieved using a 1mL Pasteur Pipette. Cells were then cleared of RBCs contamination using RBC lysis buffer and washed with PBS twice.

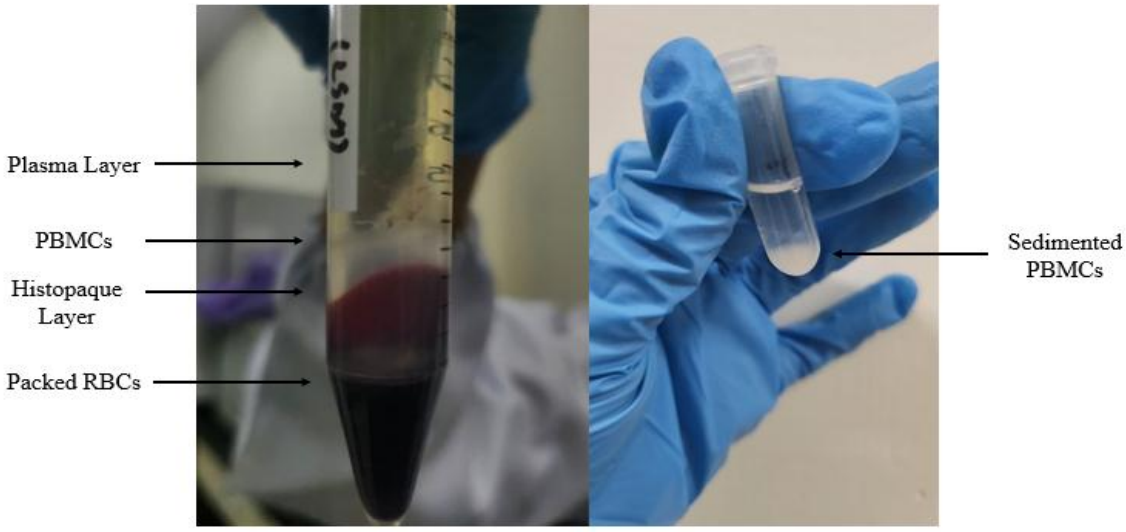

Figure 4. 1 'Isolation of PBMCs'

A) Isolated PBMCs from peripheral blood. Arrows show fractionation of blood into different layers upon centrifugation. B) White buffy layer at the interface shows population of PBMCs.

4.2.2 Cell Counting

A hemocytometer was used to count the cell, based on Trypan blue exclusion assay. A suspension of cells and dye was prepared by diluting 10µl of cells and 10µl of trypan blue [dilution factor=total volume/dilute volume (20/10=2)]. The suspension was dispensed into

grid of the hemocytometer. A microscope was used to focus at 20X objective. A tally-counter was used to count the cells in four corner squares and a central square. Dead cells dyed black by the dye weren't counted. Average of cell count was recorded for 1mL by the following formula.

For volume equivalent to 1 mL =

$$\frac{\text{No of cells in each chamber (A+B+C+D+E)}}{5}$$

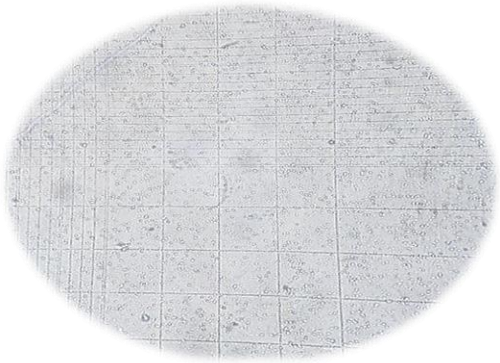

Figure 4. 2 'A Chamber of Hemocytometer.'

A single chamber of hemocytometer comprising 16 squares used for counting PBMCs by trypan blue exclusion assay. The microscope resolution was set at 20X.

4.2.3 MTT Cell viability assay

Cells were plated in 96 well plate in a dose dependent manner with following doses 10µM, 20µM, 30µM, 40µM and 50µM. It was done to study the optimal time and dosage of Sulforaphane for both healthy PBMCs and HCV infected cells. Separate plates for 24 hours and 48 hours incubation were prepared. After 24 hours and 48 hours of incubation, both control and viral infected cells were assessed under light microscope at 20X magnification. The drug induced cells appeared almost fully confluent leading to proliferation of lymphocytes and monocytes.

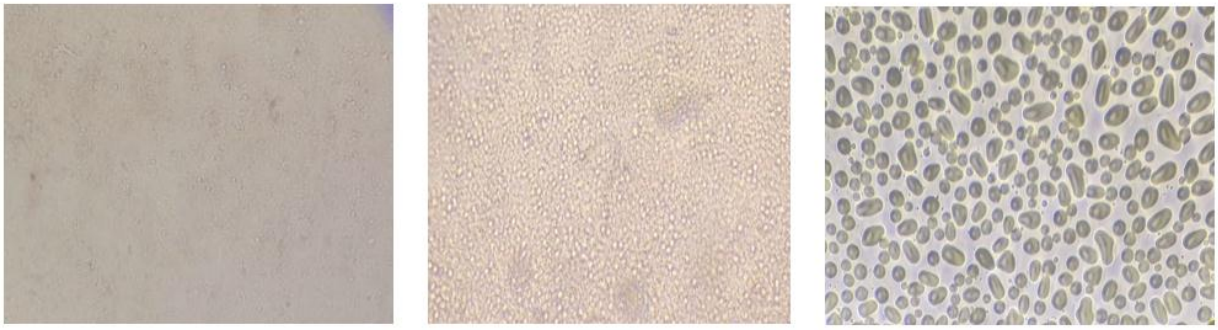

Figure 4. 3 'Morphology of Healthy PBMCS'

A) Unstimulated PBMCs of Healthy Patients. B) Drug induced at 20µM for 24 hours of incubation at 20X magnification. C) Stimulated PBMCs at 40X magnification.

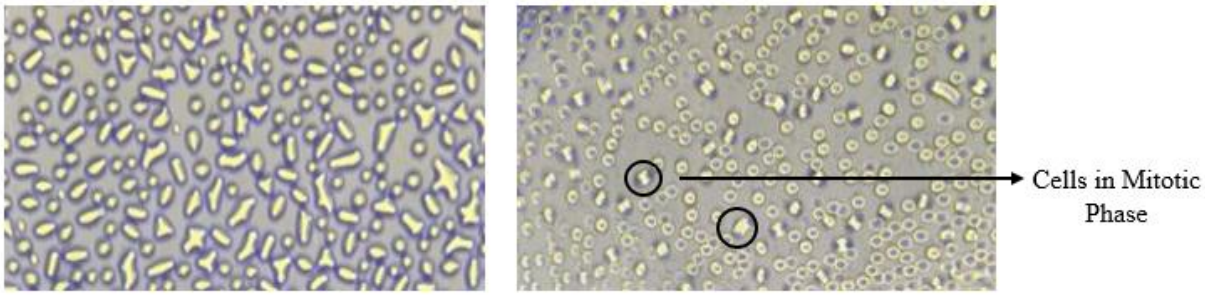

Figure 4. 4 'Morphology of PBMCs of HCV infected patients.'

A) Unstimulated PBMCs of HCV patients after 48-hour incubation period. B) Drug induced PBMCs of HCV infected patients at a dose of 20 µM after 48 hours.

A relation between absorbances and doses of R-SFN was made to evaluate the affect of drug induction and determine the optimal dose.

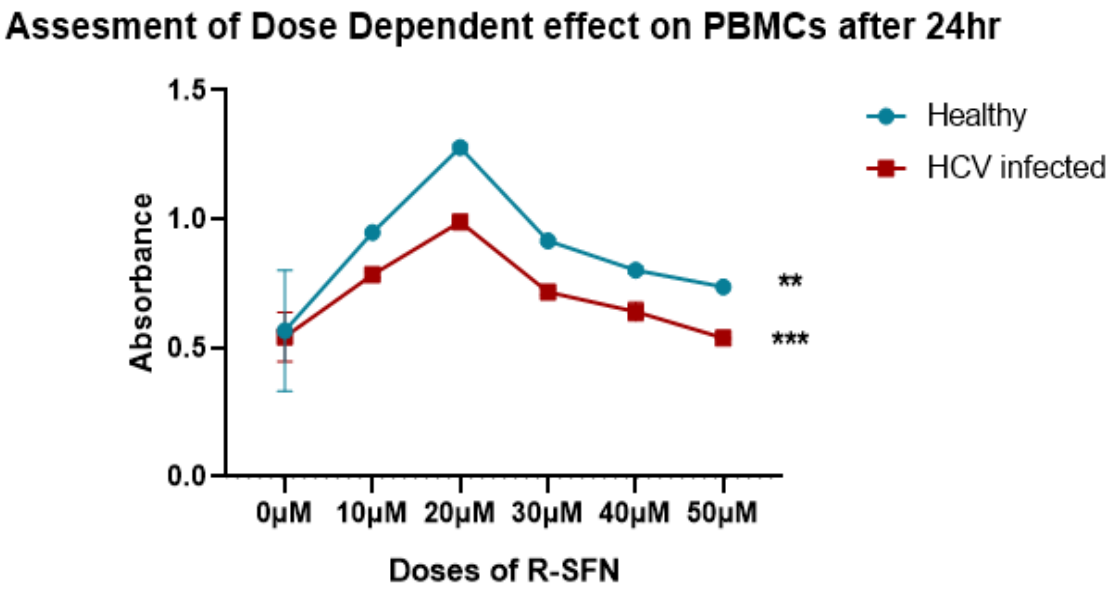

Figure 4. 5 'Assessment of Dose dependent effect of PBMCs after 24 hours.'

Assessment of various doses of R-SFN on the absorbance of PBMCs of healthy and HCV patients. Trend shows a significant difference between the absorbance levels of untreated PBMCs, and the one treated with a 20μM dose of R-SFN. A significant difference was observed in the case of experimental groups and data was analyzed by applying two-way Anova. **P <0.01, ***P <0.001 showed different levels of significance. All experiments were performed in triplicates.

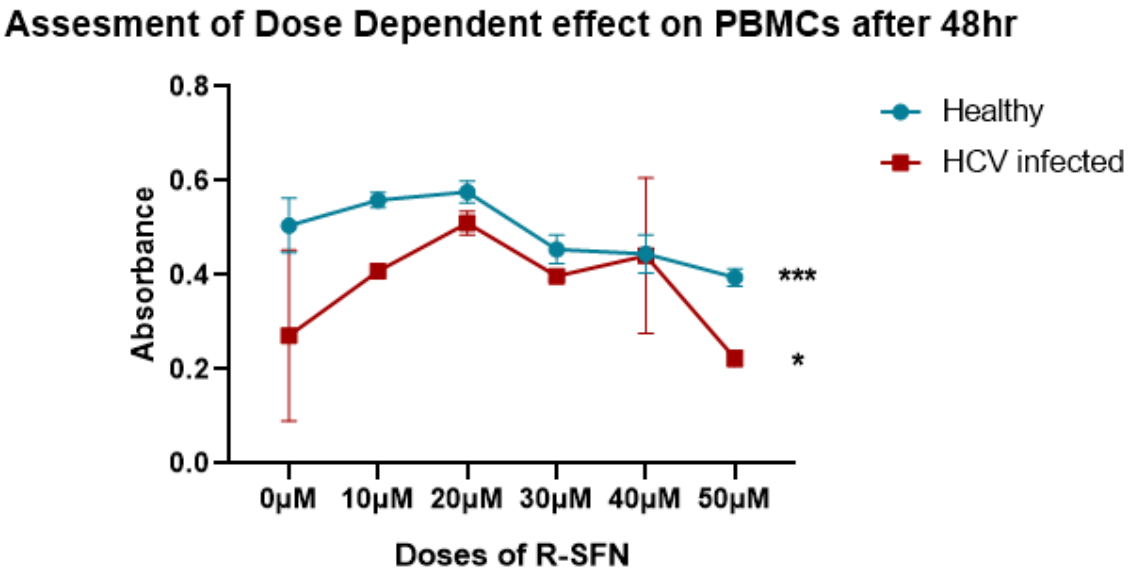

Figure 4. 6 'Assessment of dose dependent effect on PBMCs after 48 hours'

Assessment of effect of various doses of R-SFN on PBMCs of healthy and infected patients after an incubation of 48hrs. Trend shows a significant difference between the absorbance levels of untreated PBMCs, and the one treated with a 20μM dose of R-SFN. A significant difference was observed in the case of experimental groups and data was analyzed by applying two-way Anova. *P <0.05, ***P <0.001 showed different levels of significance. All experiments were performed in triplicates.

4.2.4 PBMCs culturing and Drug Stimulation

For culturing isolated PBMCs, RMPI 1640 media (10% FBS and 1% Pen/strep) was used. 4 4x10^6 were plated in 2 ml media per well in 6-well plate and stimulated with Sulforaphane in a concentration of 20 μM/ml. An incubation of 24 hours with the

parameters of 5% CO2 and 37˚C temperature. PBMCs are suspended cells and are not attached to the surface.

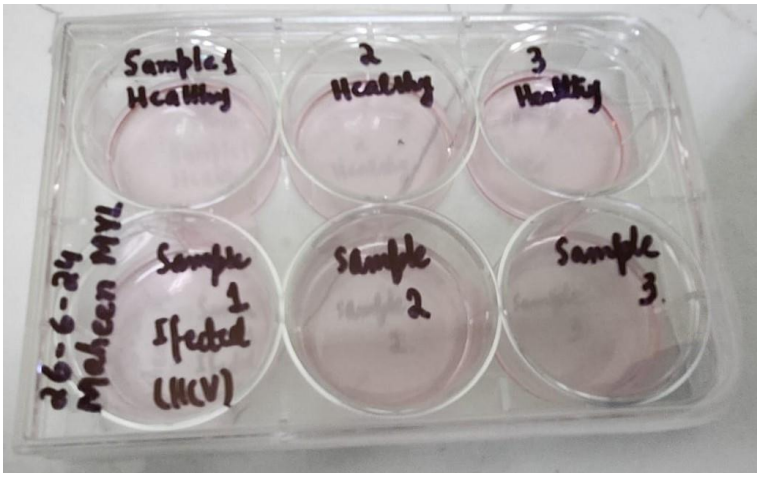

Figure 4. 7 'Culturing of PBMCs.'

Culturing of PBMCs at optimized dose of 20µM incubated for 24 hr. in 6 well plate.

4.2.5 Percent Cell Viability Assessment

According to the prescribed formula, the percentage of viable cells in the cultured healthy PBMCs treated with 20µM/mL of R-SFN is 24.33% more than untreated PBMCs.

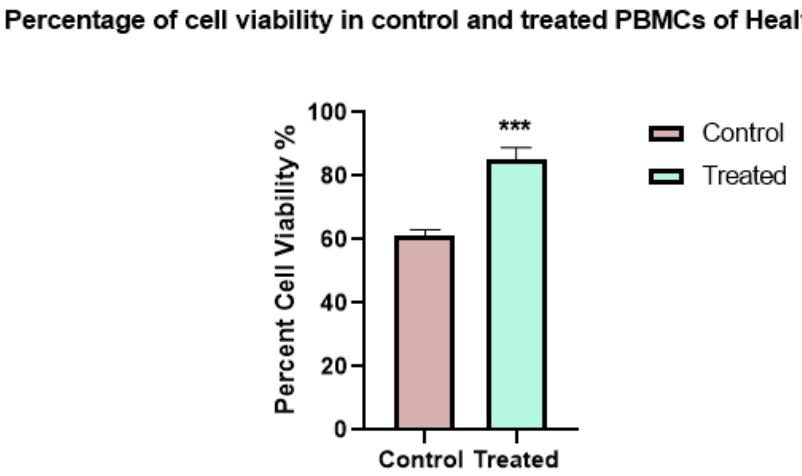

Figure 4. 8 'Percent cell viability of Healthy subjects.'

Percentage of cell proliferation in control and treated PBMCs. Treated PBMCs were significantly more viable than untreated PBMCs. Graphical representation shows that mean of treated PBMCs is significantly higher with a p-value of <0-.001 and a 95% CI of 120.4-145.9

In a similar fashion, using the same formula it was estimated that the percentage of viable cells in the cultured PBMCs of HCV infected patients treated with 20µM/mL of R-SFN is 21.23% more than that of untreated PBMCs.

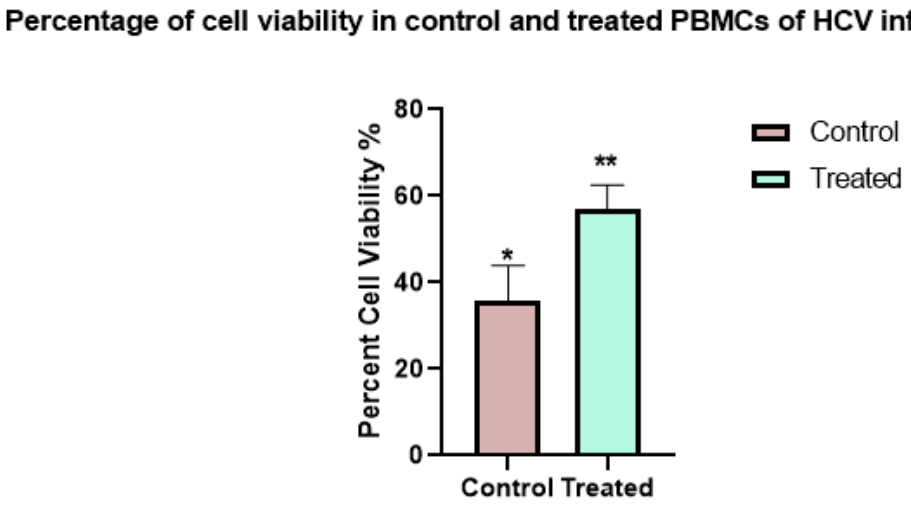

Figure 4. 9 'Percent cell viability of HCV infected subjects.'

Percentage of cell proliferation in control and treated PBMCs. Treated PBMCs were significantly more viable than untreated PBMCs. Graphical representation shows that mean of treated PBMCs is significantly higher with a p-value of <0-.005 and a 95% CI.

4.3 Extraction of RNA and cDNA synthesis

The TRIzol® method was used for extraction of RNA from PBMCs and its quantity and purity were quantified by using Nanodrop. The extracted RNA was then reverse transcribed to cDNA Synthesized c-DNA was quantified using Nanodrop, and a second validation was made by PCR amplification using housekeeping gene (GAPDH) which is consecutively always expressed in all cells.

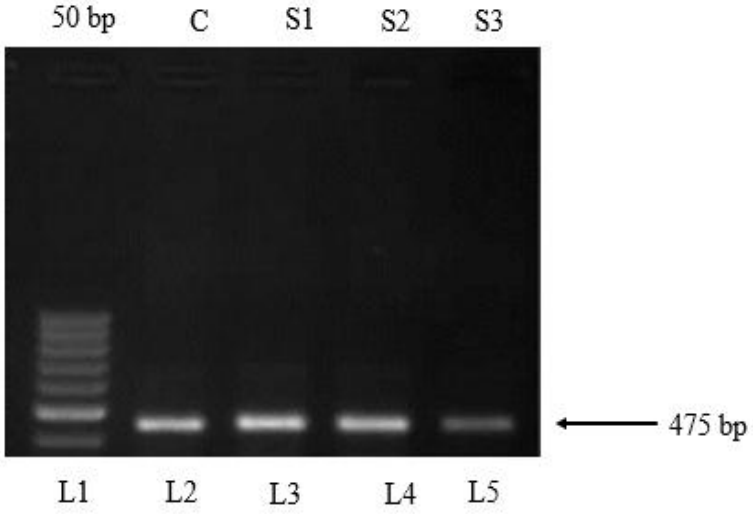

Figure 4. 10 'Integrity of cDNA.'

Integrity of cDNA by PCR amplification of housekeeping gene (GAPDH) gene was confirmed using ethidium bromide stained 1% gel electrophoresis. Bands of 475bp which appeared cleared were observed in all samples from Lane 2-5 along with 50bp DNA ladder on extreme right (L1).

4.4 Usage of conventional PCR to optimize the Amplification Conditions

The product size of the amplicon and the melting temperatures are validated using PCR (polymerase chain reaction). For the optimization of the primers different melting temperatures were tested using gradient PCR and the Tm which produced the best result for all the primers was determined. The chain reaction was carried out in a thermocycler and the amplicons were analyzed and visualized on 2% agarose gel.

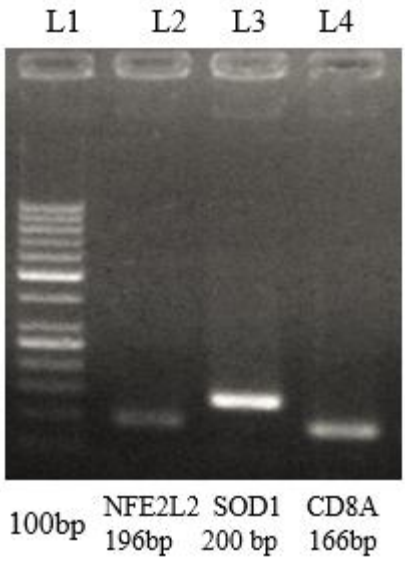

Figure 4. 11 'Primer optimization.'

Bio-Rad gel doc system was utilized to capture a gel electrogram of optimized primers. c-DNA was used as a template. Lane 1-3 show amplicons of NFE2L2 (196 bp), SOD1 (200 bp) and CD8A (166 bp). Lane 1 shows open ladder of 100 bp.

4.5 Quantitative PCR results to study the gene expression

4.5.1 Effect of R-SFN on NRF2 transcription factor

Effect of dose of R-SFN on the genes of antioxidant response element, NRF2 signaling pathway was studied. Quantitative expression of m-RNA levels was assessed in healthy as well as experimental groups (samples from patients infected with HCV) 24 hours post stimulation with -SFN. Since optimal dose was already predicted using MTT cell viability assay, all the sample were treated with a preset dose of 20 µM/ml for 24 hours. It was observed that there was difference in expression levels of treated and non-treated samples for both healthy and HCV infected patients.

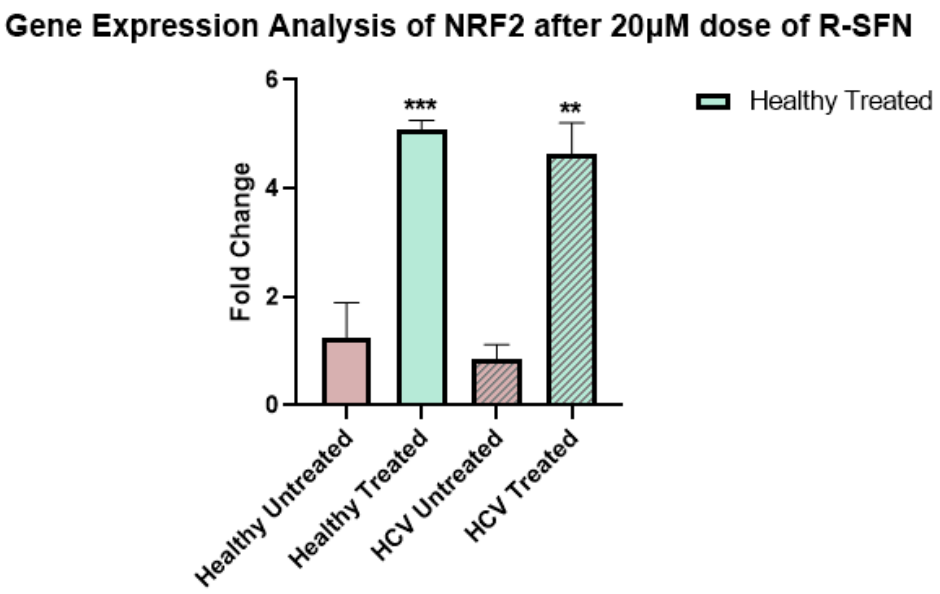

Figure 4. 12 'Gene expression of NRF2.'

Transcriptional analysis shows that there is an increase in fold change by 3.823 and 3.80 respectively for healthy and HCV infected patients for NRF2 transcription factor. Data was analyzed by applying one-way Anova and Wisconsin test. **P <0.005 and ***P <0.001 show differences in significance.

4.5.2 Effect of R-SFN on SOD-1

After investigating the genetic expression of NRF2 post R-SFN treatment, it was also studied whether R-SFN influences antioxidant gene Super oxide dismutase SOD-1. It is an anti-oxidative gene whose expression is significantly increased in PBMCs as an important standard of T cell apoptosis and dysfunction. It was observed that after 24 hours' time interval the transcriptional expression of SOD-1 was increased significantly.

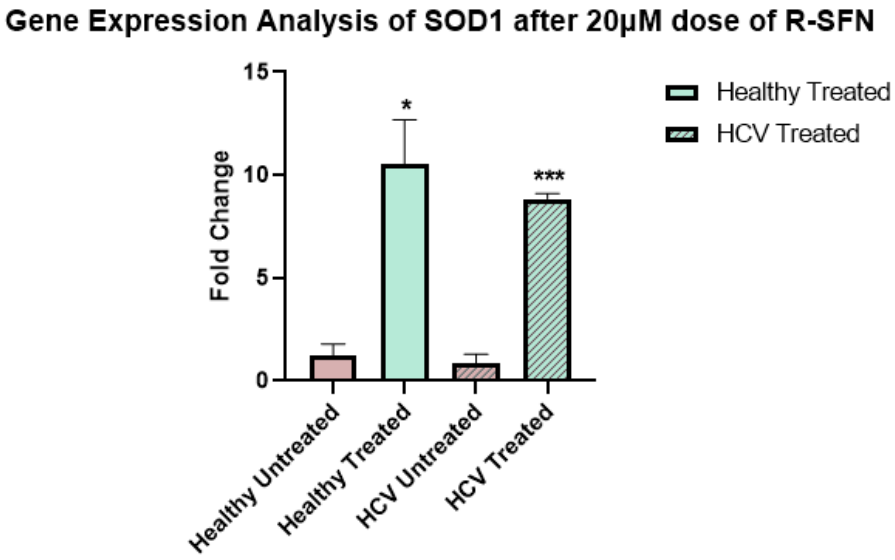

Figure 4. 13 'Gene expression analysis of SOD-1.'

Transcriptional analysis shows that there is an increase in fold change by 9.3 and 8.0 respectively for healthy and HCV infected patients for SOD1 transcription factor. Data was analyzed by applying one-way Anova and Wisconsin test. *P <0.01 and ***P <0.001 show differences in significance.

4.5.3 Effect on Sulforaphane on CD-8 T cell receptor

Real time PCR was also used to investigate whether R-SFN influences CD8A receptor of CD8 + T cells. As it is an important identity mark of T cell dysfunction and exhaustion. It was observed that transcriptional expression of CD8A was significantly increased after 24 hr post culture with R-SFN.

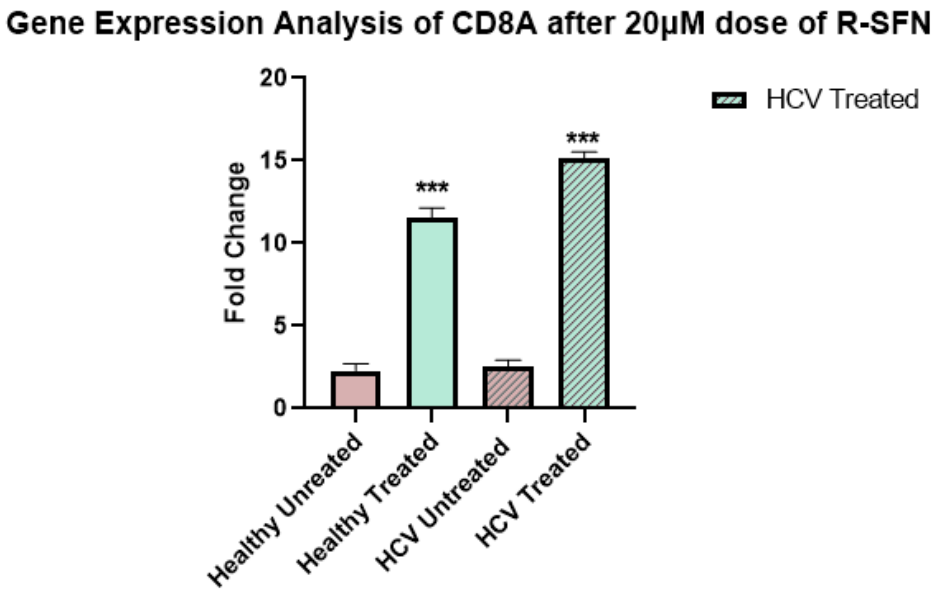

Figure 4. 14 'Gene expression analysis of CD8-A'

Transcriptional analysis shows that there is an increase in fold change by 9.3 and 8.0 respectively for healthy and HCV infected patients for CD8A chain of CD8 + T cell receptor. Data was analyzed by applying one-way Anova and Wisconsin test. ***P <0.001 show significance in results.

4.6 Docking

For the generation of docking MOE software was used. The interactions of docked template of Kelch domain of KEAP-1 was checked with KEAP-1 inhibitors.

Docking of Kelch domain with identified hits

The most active candidate among the library of 30 compounds. The compounds portrayed interactions. ChemblID was CHEMBL52277720. It showed interactions with Arg 483.

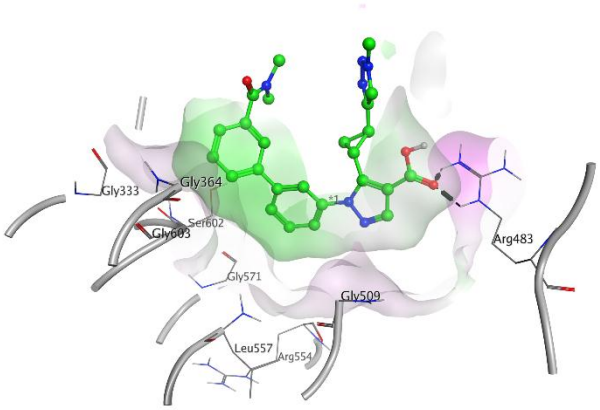

Figure 4. 15 'Docked template of Kelch domain of KEAP-1 with Active.'

4.7 GRIND

The correlogram generated of the PLS model is displayed in the figure along with the model statistics which were Q^2 value of 0.87 and R^2 value of 0.9

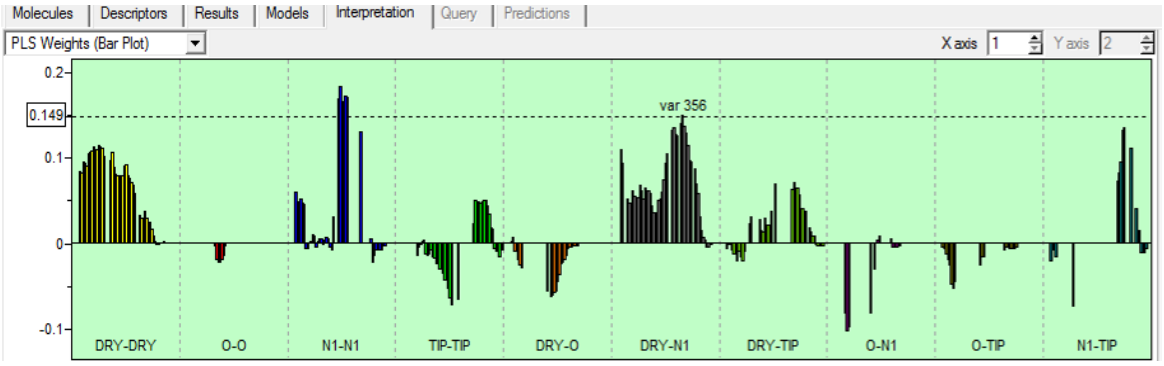

Figure 4. 16 'Correlogram of the generated model.'

CHAPTER 5: DISCUSSION

More than 200 million people in the world are currently infected with hepatitis C infection. On the social, health, economic and demographic sectors it causes an everlasting stress. In first world countries like America, Japan and Australia the disease occurring with a reduced incidence. In countries like Pakistan, and other low-income countries the occurrence is still high and gruesome. Around 6.8% of the population of the country and currently affected by this deadly virus.

It is reported in literature that chronic HCV infection can cause a failure and apoptosis of peripheral blood cells, this is directly involved in the virus to persist cause disease progression. If the virus is not resolved at proper time, it can specifically cause complete deletion of virus specific CD8+ T cells. The causative agents for this are positive antigenic stimulation, upregulation of death associated receptors. On the other hand, this is coupled with increased expansion of regulatory T cells. There is increased evidence that the apoptosis of peripheral blood mono-nuclear cells is the leading cause of diminished immunity in the chronically hcv infected individuals.

Sulforaphane is a widely and extensively known immunomodulator, anti-inflammatory and anti-apoptotic drug which also contains tested anti-cancerous properties. It has two forms R-SFN (sulforaphane) and D-SFN, both of then differ in stoichiometry and a result provide different functions. R-SFN is naturally occurring isothiocyanate which is derived from broccoli and can be seen to be anti-apoptotic towards failing and exhausted T cells and peripheral blood mono nuclear cells. These virus specific cells are noted to be functionally and structurally failing during chronic viral infections. So, one of the aim of this study will

be to critically investigate the cytoprotective effect of Sulforaphane on its venture to rescue PBMCs from apoptosis and functional exhaustion caused because of chronic HCV infection.

The first and most important aim of this study was to study the potential of sulforaphane to regulate the genes of ARE pathway which are the cause of intrinsic apoptosis of T cells. It was previously established that Sulforaphane had an anti-apoptotic nature and plays a role in balancing the number of healthy and cancerous cells by killing the cancerous cells at generally higher doses. From the results of our present study, we can confidently claim that sulforaphane played a part in supporting viral hit immunology by rescuing the PBMCs specifically lymphocytes from programmed cell death. It was noted that in ex-vivo study Sulforaphane could increase the gene expression of transcription factor NRF-2 which then along the path increased the expression of SOD-1 which is an anti-oxidant factor produced by ARE genes as well as CD-8+ T cells specific receptor CD8-A chain which proves that sulforaphane has association to increase the proliferation of CD-8 T cells (cytotoxic T cell) concentration.

All these results were seen when PBMCs were treated with an optimal dose of 20 microM for a time frame of 24 hours in the humidified incubator with 5% CO2 and at 37-degree Celsius temperature.

Another interesting aim of this study was to study signaling pathways of KEAP-1/ARE/NRF2. As NRF-2 binds to the kelch domain of the KEAP-1 As Sulforaphane binds to KEAP-1 it competes with NRF-2 and this way KEAP-1 which is deregulator of NRF2 transcription factor. It sequesters it and stops it from performing its function by

translocating into the nucleus and performing its function. When sulforaphane binds to KEAP-1 NRF2 is free to translocate to the nucleus and activate the genes of the ARE. The data extracted from the experimentation strongly suggests that Sulforaphane has a distinctive role in supporting immunity in such a way that cannot be mimicked by cytokines or DAAs. The trypan blue exclusion assay which made it possible to stain the live PBMCs among the complete population of PBMCs gave an interesting finding that Sulforaphane treated cells are more viable and even proliferating. This also supports that sulforaphane treatment stops the programmed cell death of peripheral blood mononuclear cells. As a results our hypothesis is strengthened that Sulforaphane is a strong candidate and promotes the survival factor for lymphocytes.

CONCLUSION AND FUTURE PROSPECTS

To find the exact percentage of thriving T cells in the specimens of sulforaphane treated chronic HCV patients FACS assay can be performed by using anti- CD8 anti body and anti- PD-1 anti-body to count the exact number of apoptosed cells in both treated and untreated samples so that the true numbers can be compared to have an exact idea that at what percentage the treatment with sulforaphane contributes to the niche of viral immunology. Other than that pharmacophore modeling can be used to detect used the data obtained from GRIND and apply it to predict the structure of a 3D molecule which can then be introduced to be formed on the overlap of sulforaphane, having similar physical and chemical properties but providing more robust responses mechanistically.

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APPENDICES

Appendix 1

Cell Counting

Cell count = X dilution factor x 104 cell/ml

=107.25x2x104 cell/ml

=214.5x 104cell/ml

=2.14x 106cell/ml

95% cells viability was determined by dividing the live cell count (unstained by trypan blue) from the total cell count [2.14x 106cell/ml/2.26x 106cell/ml=95%).

Appendix 2

Stock Solution of Sulforaphane

Total Concentration of Sulforaphane in vial = 10 mg

Molecular mass of Sulforaphane in 1M = 177.28 g/mol

Molecular Mass of sulforaphane in 10 mM per liter = 1.7728 g

$$C1V1 = C2V2$$

$$(1.7728g)(1000mL) = (0.01g)(V2)$$

$$V2 = 5.643\ mL$$

5.643 mL of DMSO added to 10 mg of R-SFN, initially stored at -20 ˚C and then transferred to -80 ˚C for long term storage

Appendix 3

Dose Calculations of Sulforaphane

For creating an optimal dose of 20 µM in triplicate =

$$C1V1 = C2V2$$

$$(10mM)(x) = (20)(300)$$

$$(10000)(x) = (20)(300)$$

$$0.9\ \mu L$$

Appendix 4

Preparation of RPMI 1640 media

RPMI 1640 media was supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic (Penicillin and streptomycin).

Appendix 5

1X PBS

1000mL of 1 X PBS was prepared by mixing the following, NaCl =

8g

KCl = 0.2g, Na2HPO4 = 1.44g and KH2PO4 = 0.24 g

Making the volume up to 1000mL and adjusting pH to 7.4

Appendix 6

RBC lysis buffer

NH_4Cl = 17.98g, $KHCO_3$ = 2g and 0.5M EDTA = 400mL

Dissolve in 120mL distilled water, Qs up to 200mL, maintain pH at 7.3.

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Dr. Sobia Manzoor, PhD
Professor (Tenured)
Dept. of Healthcare Biotechnology, ASAB
National University of Sciences and Technology (NUST), Islamabad

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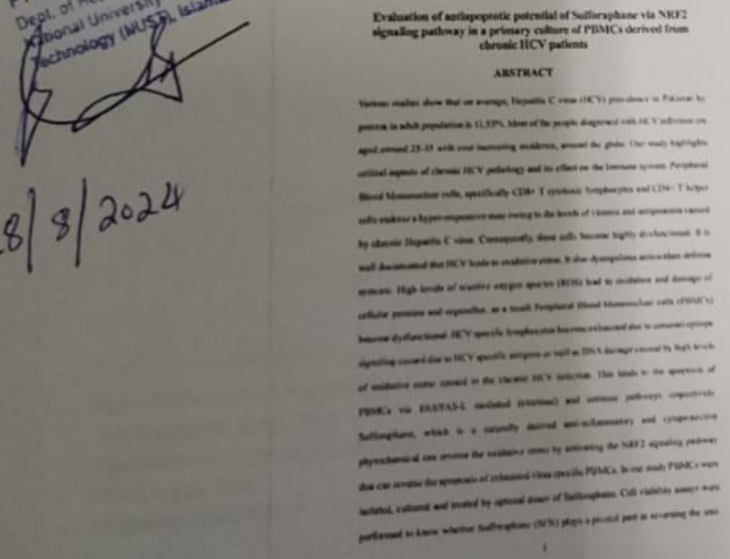

Evaluation of antiapoptotic potential of Sulforaphane via NRF2 signaling pathway in a primary culture of PBMCs derived from chronic HCV patients

ABSTRACT

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