INTRODUCTION

VIRUSES

Viruses are subcellular, infectious agents that are obligate intracellular parasites. This is a fundamental characteristic of all viruses. They infect and take over a host cell in order to replicate. The mature, extracellular virus particle is called a virion. The virion contains a genome that may be encoded as DNA or RNA, wrapped in a protein coat called a capsid or nucleocapsid. Some viruses have a lipid envelope surrounding the nucleocapsid. In such viruses, glycoproteins encoded by the virus are embedded in the lipid envelope. The functions of the capsid or envelope are to protect the viral genome while it is extracellular, and to promote the entry of the genome into a new cell [1].

In mammalian cells, the information of the genes is encoded in DNA. Such information is expressed via messenger RNA (mRNA) which is translated in the cytoplasm of the cell by ribosomes into proteins. All viruses must direct the synthesis of mRNA to produce proteins, as viral protein synthesis is completely dependent on the host cell's translational machinery.
By convention, mRNA is defined as a positive (+) strand because it contains immediately translatable information. The RNA and DNA complements of (+) strands are designated as negative (-) strands [2].

HEPATITIS VIRUSES

The hepatitis viruses are pathogens that affect millions of people around the world. These viruses cause inflammation of the liver and therefore, were mainly identified for their abilities to cause transmissible hepatitis in humans.
Many different viruses, belonging to several viral families, are known to cause hepatitis in man. These distinct viruses have different modes of transmission and cause illness of

varying degrees of severity. The illness results from destruction of liver cells caused by growth of these viruses in the liver as a target organ.

The viruses whose primary disease syndrome in man is hepatitis, or which are closely related to viruses that cause such hepatitis, have been designated hepatitis virus followed by a letter, assigned in the order of isolation. Because these viruses belong to a number of different families, it can be confusing due to the similarity of the names, even though the viruses are unrelated [1]. Table 1 presents a description of the currently known hepatitis viruses [1, 3].

Table 1. Characteristics of Hepatitis Viruses A-G

(# Family; * Genus, + Not further characterized)

Viruses	A[1]	B[1]	C[1]	D[1]	E[1]	F[3]	G[3]
Classification	*Picornaviridae*[#]	*Hepadnaviridae*[#]	*Flaviviridae*[#]	*Deltaviridae*[*]	*Unclassified*	*?*[+]	*Flaviviridae*[#]
Genome	RNA	DNA	RNA	RNA	RNA		RNA
Envelope	No	Yes	Yes	Yes	No		Yes
Primary source	Feces; food/water-borne	Blood and blood products	Blood and blood products	Blood and blood products	Feces; water-borne		Blood/sexual fluids
Primary mode of transmission	Fecal-oral	Parenteral	Parenteral	Parenteral	Fecal-oral		Parenteral/sexual
Type of hepatitis	Acute hepatitis	Acute and chronic hepatitis	Acute and chronic hepatitis	Acute and chronic hepatitis	Acute hepatitis		Acute and chronic hepatitis

[1] Strauss, J., Strauss, E. Viruses and Human Disease 2002. Academic Press, London, UK.
[3] Ou, J.H.J. Hepatitis Viruses.2002. Kluwer Academic Publishers. Massachusetts, USA.

HEPATITIS C VIRUS

The hepatitis C virus is part of the Flaviviridae family [4]. The Flaviviridae family is divided into three genera, the genus *Flavivirus* (Dengue fever; Yellow fever; Japanese encephalitis; St. Louis encephalitis; Murray Valley encephalitis, and Tick-borne encephalitis), the genus *Pestivirus* (Classical swine fever and Bovine viral diarrhea), and the genus *Hepacivirus* (Hepatitis C, HCV). The genomes of the three genera are similar in size (11 kb for flaviviruses, 12.5 kb for pestiviruses and 9.4 kb for hepaciviruses) and organization [1]. These viruses have a genome that contains a single open reading frame (ORF). The structural proteins are encoded in the 5'-terminal region of the viral genome. All three genera are enveloped and the structural proteins consist of a nucleocapsid protein and two or three envelope glycoproteins. Cellular proteases make the cleavages that separate the glycoproteins, but the cleavages in the nonstructural region of the polyprotein, which are required for RNA replication, are made by one or two viral-encoded proteases [1].

The causative agent, previously designated non-A, non-B hepatitis virus and now referred to as HCV, was identified in 1989 [5]. The size of infectious virus is 30 to 80 nm in diameter, its genome is a 9.6-kilobase positive single-strand RNA (ssRNA) molecule. Figure 1 shows the HCV proteins encoded by the open reading frame of the HCV genome.

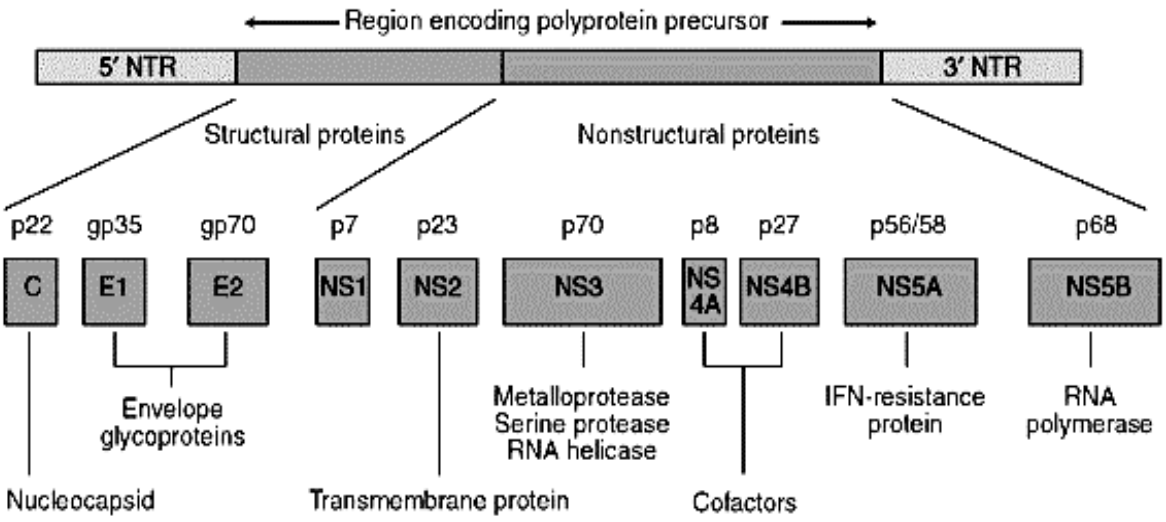


Figure 1. HCV Genome Organization[*]

[*] Anzola and Burgos. Expert Reviews in Molecular Medicine. 2003. Cambridge University Press, UK.

HCV contains both 5' and 3' non-translated regions (NTRs). The NTRs flank an open reading frame which encodes a single large polyprotein of around 3010 amino acids. The polyprotein is processed into structural proteins: Core (C), two putative envelope proteins E1 and E2, and a short protein called p7. These N-terminal proteins are thought to be necessary for forming the complete infective viral particle (virion). The nonstructural region encodes for proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. Some of these are cleaved into smaller units and are thought to be involved in the replication of the virus within the cell [6]. The cleavages of the structural proteins are catalyzed by a host signal peptidase localized in the endoplasmic reticulum (ER). The nonstructural proteins are processed by two virus proteinases, NS2 and NS3. The region NS5B encodes a protein which has RNA-dependent RNA polymerase activity [7].

The 5' NTR functions as an internal ribosomal entry site (IRES), allowing the initiation of polyprotein translation [8]. The 3' NTR has a stimulatory effect on HCV IRES-mediated translation, and also it is probably important for the initiation of RNA replication [9].

HCV is classified on the basis of similarity of nucleotide sequence into major genetic groups called genotypes. HCV has six genotypes which vary in nucleotide sequence by 30 to 50% [10]. In the United States, the most common is genotype 1 (approximately 75% of cases), followed by genotype 2 (approximately 15%) and genotype 3 (approximately 7%). The six distinct genotypes of HCV show marked differences in geographic distribution, disease progression and response to therapy.

EPIDEMIOLOGY

Approximately 170 million people around the world are infected by hepatitis C virus (HCV) (Table 2) [11]. Of these people, a substantial proportion are at risk of hepatocellular carcinoma.

The group most at risk of developing hepatitis C is intravenous drug abusers. Transmission via inadequately sterilized needles, body piercing, tattooing, and circumcision have also been implicated. Sexual exposure and congenital infections are less frequent. Organs transplants have also transmitted HCV infections.

Table 2. World Prevalence of Hepatitis C Infection

World Health Organization	Hepatitis C Prevalence (Percent)	Infected Population (Millions)
Africa	5.30	31.9
Americas	1.70	13.1
Eastern Mediterranean	4.60	21.3
Europe	1.03	8.9
Southeast Asia	2.15	32.3
Western Pacific	3.90	62.2
Total	Average= 3.1	Total= 169.7

[11] Collier, L., Oxford, J. Human Virology 2006. Oxford University Press, UK.

CLINICAL FEATURES

The incubation period of HCV is about 8 weeks. Not all the people present initial symptoms like anorexia and nausea. When the jaundice occurs, levels of serum alanine and aspartate aminotransferases levels begin to increase shortly before the presentation of symptoms.

Hepatitis C becomes chronic in about 80% of infected people. After many years (around 3-4 decades), cirrhosis of the liver may occur in 20-30% of patients. The development of cirrhosis is sinister because it is often a precursor of hepatocellular carcinoma, which develops in 1-5% of patients with chronic hepatitis C (CHC).
The chronic infection is silent, and may be discovered only in routine testing such as during blood donation, routine health screening or evaluation for an unrelated condition.
In CHC, damage and death of infected hepatocytes may be caused directly by the virus damaging the cells, or indirectly by immune responses of the host. Chronic hepatitis C leading to hepatocellular carcinoma (HCC) is the most common cause for liver transplantation in people with end-stage liver disease.

HCV REPLICATION

The virus replicates in the liver at a high rate, producing an average serum HCV RNA level of 1 to 2 million genome equivalents per milliliter [12].

The precise molecular mechanism of HCV replication is not known, however it is thought that is similar to the replication of other positive stranded RNA viruses.
Virus particles bind to a receptor on a surface of the cell membrane. The cell membrane then surrounds and pinches off the virus particles, resulting in entry into the cell. After the viral particle is uncoated in the cytoplasm of the host cell, the viral genome acts as a template for the synthesis of a complementary RNA molecule (- RNA). This minus-

strand serves as a template for the synthesis of new positive stranded RNA molecules (progeny). Figure 2 shows a model of the HCV replication system [13].

Initially, HCV is incorporated into human liver cells. The internalized virus then dissociates, liberating the viral RNA genome. The HCV RNA is then translated by host ribosomes, producing the HCV polypeptide. This polypeptide is subsequently processed, first by host peptidases and then by the HCV proteases (NS2 and NS3) into the ten different HCV proteins. The nonstructural (NS) proteins are assembled and localized within the liver cell to form a replication complex which produces multiple copies of the HCV RNA genome. The HCV nonstructural proteins NS3 to NS5B direct viral replication to form a ribonucleoprotein replication complex associated with an ER-derived membranous web [14]. These RNA copies are then able to enter to the cycle again, producing more HCV proteins. The HCV structural proteins (C, E1 and E2), along with copies of HCV RNA, are packaged as infectious virus particles, released from the liver cell, and are then able to infect new cells [15].

Although little is known about the mechanisms and host functions involved in viral entry, uncoating, trafficking, assembly and egress, the recently developed *in vitro* HCV infection system, JFH-1 in Huh7.5 cells, should make these processes experimentally accessible.

In the main, HCV does not kill the cells it infects, but triggers an immune-mediated inflammatory response (hepatitis) that either rapidly clears, or slowly destroys the liver, sometimes causing the development of hepatocellular carcinoma.

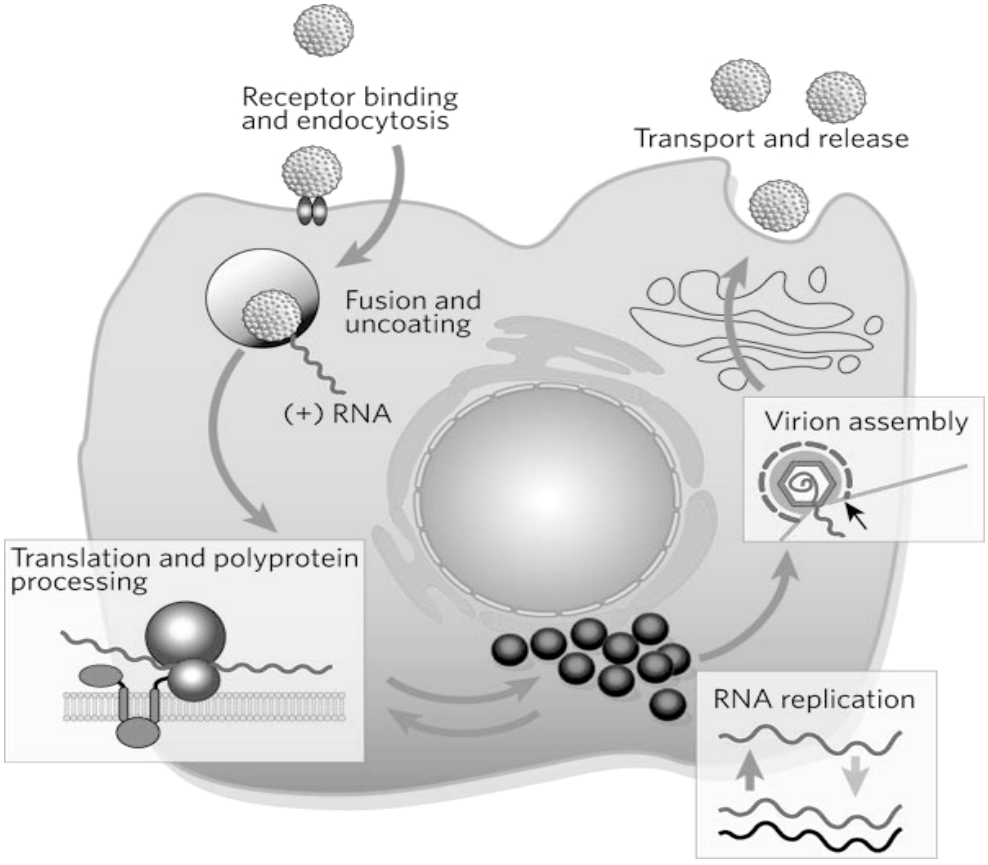


Figure 2. HCV Life Cycle[13]

[13] Brett D. Lindenbach and Charles M. Rice. Unravelling hepatitis C virus replication from genome to function. Nature 2005; 436: 933-938.

HCV THERAPY

Most HCV infections become chronic and for around 50% of patients this condition is gradually, progressive, perhaps, leading to cirrhosis, end-stage liver disease and hepatocellular carcinoma. The current standard therapy for chronic hepatitis C is a combination of pegylated interferon (IFN) and ribavirin, but the response to the treatment varies depending on viral and host characteristics, especially the viral genotype. The combination therapy is moderately effective in ~50% of people with chronic hepatitis C, but many patients fail to respond to/or relapse following treatment.

Interferon α and β (IFN-α and β) are cytokines that have an important function in the innate antiviral immune response. They attach to the cell surface receptors that signal through the Janus-activated kinase and signal transducers and activators of transcription (JAK-STAT), leading to induction of multiple interferon stimulated genes. Such genes include double-strand RNases, inhibitors of viral protein translation, and proteins that destabilize viral messenger RNA. Ribavirin is a nucleoside analogue with a broad activity against viral pathogens, used alone, ribavirin is insufficient for therapy, but in combination with IFN there is synergistic antiviral effect. Nevertheless, this current standard combination therapy for chronic viral hepatitis remains unsatisfactory in many patients. The virological response rates with pegylated IFN-α and ribavirin in patients with hepatitis C are about 54-63% [16].

In addition to the high cost of the treatment and its low availability in many parts of the world, this therapy has been shown to cause serious side effects. The most common are fatigue, muscle aches and psychological disorders such as depression, irritability, anxiety and sleep disturbance. For these reasons, many infected people believe that the cure is worse than the disease.

The most common adverse effects of ribavirin are haemolysis, anemia and skin rash [17].

COMPLEMENTARY AND ALTERNATIVE MEDICINE (CAM)

CAM has been known by a variety of terms like complementary, holistic, alternative, unorthodox or integrative medicine. CAM refers to most treatment practices that are not considered conventional medicine, that is, those that are not widely practiced or accepted by the orthodox medical community.
Millions of people around the world use some form of complementary and alternative medications. In the USA in 2001, around 70% of interviewed people responded that they had used CAM at least once in their lifetimes [18].

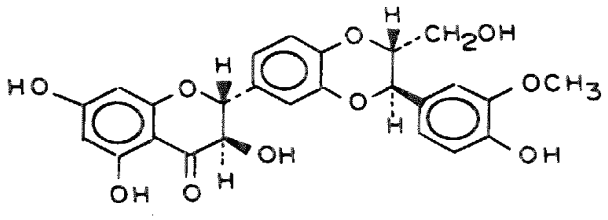
CAM as defined by the National Center for Complementary and Alternative Medicine (NCCAM) is a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine. Conventional medicine is medicine as practiced by holders of M.D. (medical doctor) or D.O. (doctor of osteopathy) degrees and by their allied health professionals, such as physical therapists, psychologists, and registered nurses [19].
HCV-infected patients often consider the use of CAM as an alternative to, or in conjunction with, IFN-α and ribavirin treatment, due to the costs of this treatment, the severe side-effects and the low efficacy of the therapy.

SILYMARIN

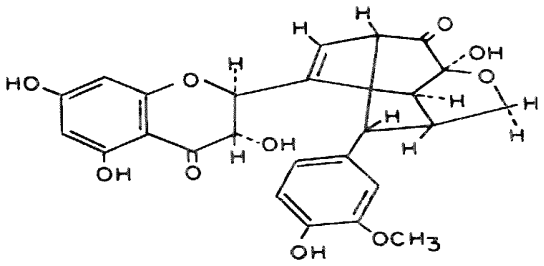
One of these alternative medications which many patients with CHC consume is milk thistle or silymarin. Silymarin is a plant flavonoid which is found and extracted from the seeds and fruits of the milk thistle *Silybum marianum* [20]; the dried seeds contain 1-4% by weight of silymarin flavonoids.

A number of therapeutic and curative properties have been ascribed to flavonoids and many of them have been incorporated into popular folk remedies. Silymarin was introduced as a “hepatoprotective” agent in 1983 [21].

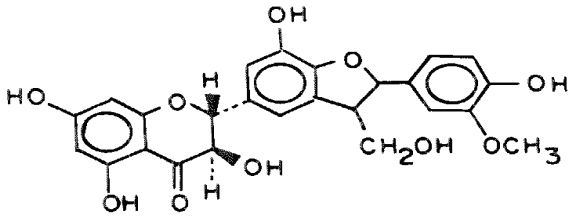
Flavonoids such as Silymarin have been used, either as such, or as part of several chemically complex preparations. Silymarin is a mixture of at least three structural isomers, silibinin, silidianin and silichristin. Figure 3 shows the structural formulas of these three isomers.



Silibinin



Silidianin



Silichristin

Figure 3. Silymarin Structural Isomers(*)

* Valenzuela, A., Garrido, A. Biochemical bases of the pharmacological action of the flavonoid silymarin and of its structural isomer silibinin. Biol Res 1994; 27: 105-112.

Most of Silymarin hepatoprotective properties are attributed to silibinin which is the main constituent (60-70%). For treatment of diseases, it is typically safe to consume 1.4 g of silymarin daily for a week, which produces silibinin levels in the blood of patients of about ~3 μmoles/L [22]. Because silymarin is poorly absorbed in the intestine, there are formulations that include a unit of phosphatidylcholine attached to it, which enhances its bioavailability.

Flavonoids are recognized as good antioxidant compounds. The presence of hydroxyl groups in different positions of their benzene rings makes hydrogen abstraction feasible, resulting in the neutralization of free radicals [23].

All aerobic organisms form or degrade reactive oxygen species (ROS), leading to physiological concentrations required for normal cell function. Excessive quantities of ROS lead to a state of oxidative stress. This state is described as the structural and/or functional damage produced in a tissue by the uncontrolled formation of prooxidant oxygen free radicals [24]. Generally, oxidative stress develops when the prooxidant action of an inducer (enzyme, xenobiotic or metal) exceeds the antioxidant capacity of the cellular defense system, surpassing its homeostatic capability and eventually leading to death.

The mechanism of injury in hepatitis C includes oxidative stress as a result of the altered mitochondrial function suspected to be a direct effect of HCV core protein [25]. Also the interaction of the HCV core protein with mitochondria, and the subsequent oxidation of the glutathione pool and complex I inhibition, can be an important cause of the oxidative stress seen in chronic hepatitis C [26]. It has been also shown that HCV NS5A protein disturbs intracellular calcium levels and triggers an increase in mitochondrial ROS formation [27]. Due to this increase, antioxidant therapy may have beneficial effects in hepatitis C patients.

The possible hepatocyte membrane stabilizing, regeneration-promoting and iron binding properties of silymarin may be of benefit in hepatitis C [28]. Overall, studies that have analyzed the possible beneficial role of silymarin in hepatitis C treatment have not reported any beneficial effects on serum viral levels. However, a recent study [29] *in*

vitro shows that silymarin exerts anti-viral and anti-inflammatory effects in liver cells, suggesting that it may assist in the management of patients with chronic hepatitis C.
The biochemical mechanism(s) of action of silymarin is (are) not yet well understood, however, it is possible that there exists a relationship between the effects of silymarin and the JAK-STAT pathway to decrease the viral load *in vitro* [29], but this still remains uncertain and more studies *in vitro* and *in vivo* would need to be done in order to prove or disprove whether such a relationship exists.

HEME OXYGENASE-1 (HO-1)

Cells are protected against oxidative stress by numerous intracellular antioxidant compounds and by diverse antioxidant enzymes. Most of these enzymes can be up-regulated by various physical, chemical, and biological agents that induce oxidative stress. One of these inducible enzymes is heme oxygenase-1 (HO-1).
HO-1 protein (~32 kDa) catalyzes the degradation of heme to release free iron and equimolar amounts of carbon monoxide and biliverdin, the latter of which in mammals is converted to bilirubin by the enzyme biliverdin reductase.

HO-1 occurs at high levels in the spleen and also in the liver parenchyma, which is the major site of uptake and degradation of plasma heme and hemoglobin. At the cellular level, HO-1 has been characterized as an endoplasmic reticulum (ER) associated protein due to the abundant detection of HO-1 activity in microsomal fractions. Other studies have raised the possibility of the functional compartmentalization in other subcellular domains beside the ER including the nucleus, mitochondria, and the plasma membrane [29a, 29b].

Although HO-1 typically occurs at very low or undetectable levels under basal conditions, it is transcriptionaly up-regulated by a large variety of stimuli, including its substrate heme, oxidative stress and other chemical or physical stimuli (heavy metals, hyperthermia, UV irradiation, and inflammatory cytokines). Most, if not all, of the

chemical and physical agents that induce HO-1 gene expression operate in a network of signaling pathways which converge on the activation of an equally complex network of transcriptional regulators [30, 31, 32, 33].

Analyses of the promoter regions of HO-1 genes from various species (mouse, rat, human and chicken) have revealed a multiplicity of response elements which serve as binding sites for transcription factors.
The heme-mediated induction of HO-1 is regulated principally by two upstream enhancers, E1 and E2 [34]. These enhancer regions contain multiple antioxidant response elements (ARE).
A multiplicity of DNA-binding proteins interact with these regulatory regions. Among these are Nrf2 and Bach1 proteins which heterodimerize with the small Maf proteins [35]

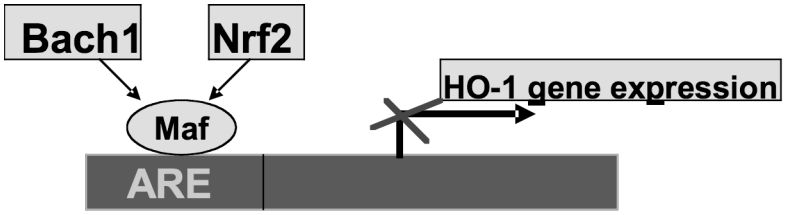
Nrf2 (NF-E2 related factor-2), is a transcription factor which recognizes and binds to consensus antioxidant response elements sequences found in the promoter regions of several phase II enzymes involved in xenobiotic metabolism, as well as to HO-1. Nrf2 has been associated with HO-1 gene activation in response to multiple agents (i.e., cobalt protoporphyrin) [30]. It forms stable heterodimers with Maf proteins family [36].

Another ARE binding factor is Bach1 which plays an important role in the negative regulation of HO-1 transcription. Bach1 also forms heterodimers with the small proteins of the Maf family. In contrast with Nrf2, Bach1 lacks a transactivation domain, and therefore Bach1 functions as a repressor when it binds to the target sequences. The repressor activity of Bach1 dominates over the activator function of other binding proteins such as Nrf2, thus maintaining effectively the gene activity at low levels under basal conditions. Bach1 also binds with high affinity to heme *in vitro*, which inhibits its *in vitro* DNA binding activity. Heme binding to Bach1 also promotes the nuclear export of Bach1 in intact cells. Thus, in the absence of heme, HO-1 gene expression is repressed by Bach1 [31]. Displacement of Bach1 from the ARE by increased levels of intracellular heme is followed by binding of Nrf2 to these elements [37], suggesting that high levels of

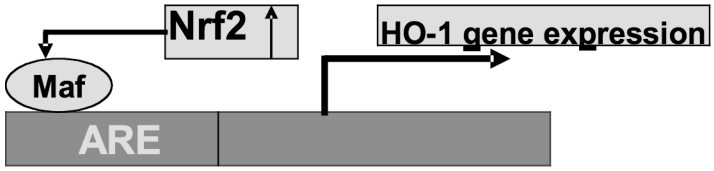
gene transcription also require the subsequent action of a ARE-binding protein with activator function, in this case Nrf2.

Figure 4 shows a schematic model of repression and induction of HO-1 via Bach1 and Nrf2 transcription factors.

Under basal conditions



Putative Silymarin HO-1 Up-Regulation



Putative Silymarin HO-1 Up-Regulation

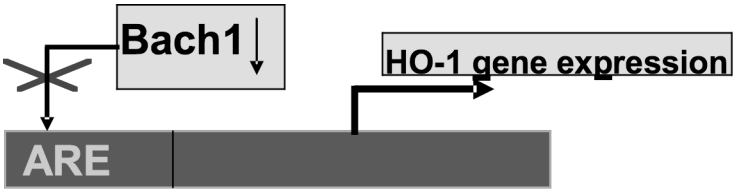


Figure 4. Schematic Model of Repression and Induction of HO-1 by Bach1 and Nrf2.

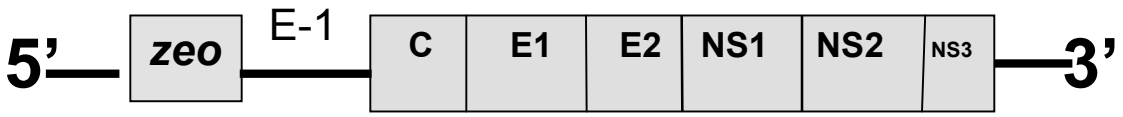
According to this model, one would expect to see a reduction in Bach1 gene expression if Silymarin up-regulates HO-1 by this transcription factor. Alternatively, one would expect an increase in the levels of Nrf2 transcription factor, if Silymarin induces HO-1 via the Nrf2 transcription factor.

IMPORTANCE OF THE REPLICONS

Molecular analyses of HCV replication have historically been hampered by the lack of convenient animal models or efficient cell culture systems that allow for the production of infectious virus particles. The difficulties may have been due to the lack of essential host cell factor(s) or to the presence of an assembly inhibitor. Also the viral genomes used in attempts to establish the replicons may have been defective, or the cell lines used may have lacked cell culture adaptive mutations that are necessary in order to achieve sufficient RNA replication [38]. Even though there has been progress in understanding the genomic organization of the virus and the functions of viral proteins, many fundamental aspects of HCV replication, pathogenesis, and persistence remain unknown. Recently, the development of selectable subgenomic HCV replicons have, for the first time, enabled the study of HCV replication in cell culture.

Figure 5 shows a schematic representation of the structures of the HCV genomes used in this study. Wild type Huh-7 cells are derived from a human liver tumor.

CNS3 cells



9-13 cells

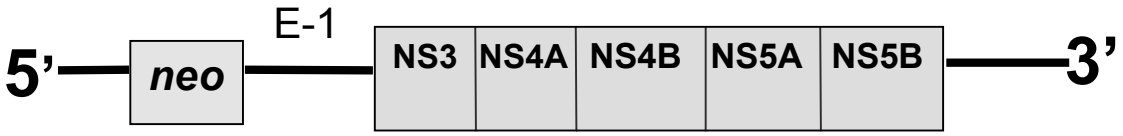


Figure 5. Schematic Representation of the HCV Genomes Used in This Project.

CNS3 cells expresses a zeo resistance gene (zeo), the IRES of the encephalomyocarditis virus (EMCV) (E-I), which directs translation of HCV sequences. 9–13 cells are composed of HCV-IRES, the neomycin phosphotransferase (neo) gene and E-1.

These replicons were derived from a full-length HCV consensus genome of genotype 1b [39].

The HCV replicon technology has several applications for studying the HCV viral life cycle, and also it has a potential impact for the clinic, the most important being the development and effects of antiviral drugs, as well as for testing drug combinations.

Another HCV genome that replicates to high levels has been developed. This system is based on a cloned genotype 2a genome, which was isolated from a Japanese patient with fulminant hepatitis (JFH-1) [40]. The JFH-1 RNA replicates at a higher rate than any of the other replicons. Also, when the full length JFH-1 genome is transfected into Huh-7 cells it leads to the production of HCV particles that are infectious in cells and in chimpanzees [41]. A study demonstrated that recombinant viruses generated in Huh7.5 cells are infectious in chimpanzees and in a mouse model, and that the viruses recovered from these animals remain infectious in cultured cells [42]. These results demonstrate that it is possible to grow HCV in cell culture.

Although it is obvious that cell lines differ from normal hepatocytes in several important aspects, the HCV replicon system simulates the basic principles of HCV replication *in vitro* as it occurs *in vivo*.