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Naringenin inhibits the assembly and long-term production of infectious hepatitis C virus particles through a PPAR-mediated mechanism

Jonathan Goldwasser^{1,2,3}, Pazit Y. Cohen⁶, Wenyu Lin³, Danny Kitsberg⁶, Patrick Balaguer⁴, Steven J. Polyak⁵, Raymond T. Chung³, Martin L. Yarmush^{1,3}, and Yaakov Nahmias^{1,3,6,*}

¹Center for Engineering in Medicine, Shriners Burns Hospital, Boston, MA, USA

²Harvard-MIT Division of Health Science and Technology, Cambridge, MA, USA

³Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

⁴INSERM, Univ. Montpellier I, Montpellier, France

⁵University of Washington, Seattle, WA, USA

⁶The Selim and Rachel Benin School of Engineering, The Hebrew University of Jerusalem, Jerusalem, Israel

Abstract

Background & Aims—Hepatitis C virus (HCV) infection affects 3% of the world population and is the leading cause of chronic liver disease worldwide. Current standard of care is effective in only 50% of the patients, poorly tolerated, and associated with significant side effects and viral resistance. Recently, our group and others demonstrated that the HCV lifecycle is critically dependent on host lipid metabolism and that its production is metabolically modulated.

Methods—The JFH1/Huh7.5.1 full lifecycle model of HCV was used to study the antiviral effects of naringenin on viral replication, assembly, and production. Activation of PPAR α was elucidated using GAL4-PPAR α fusion reporters, PPRE reporters, qRT-PCR, and metabolic studies. Metabolic results were confirmed in primary human hepatocytes.

Results—We demonstrate that the grapefruit flavonoid naringenin dose-dependently inhibits HCV production without affecting intracellular levels of the viral RNA or protein. We show that naringenin blocks the assembly of intracellular infectious viral particles, upstream of viral egress. This antiviral effect is mediated in part by the activation of PPAR α , leading to a decrease in VLDL production without causing hepatic lipid accumulation in Huh7.5.1 cells and primary human hepatocytes. Long-term treatment with naringenin leads to a rapid 1.4 log reduction in HCV, similar to 1000 U of interferon. During the washout period, HCV levels returned to normal, consistent with our proposed mechanism of action.

Conclusions—The data demonstrates that naringenin is a non-toxic assembly inhibitor of HCV and that other PPAR α agonists play a similar role in blocking viral production. The combination

of naringenin with STAT-C agents could potentially bring a rapid reduction in HCV levels during the early treatment phase, an outcome associated with sustained virological response.

Keywords

HCV; Naringenin; Lipid metabolism; PPAR α

Introduction

Hepatitis C virus (HCV) infection is a global public health concern, affecting an estimated 170 million individuals [1]. HCV infection develops into a chronic condition in over 70% of patients and is the leading cause of chronic liver disease world-wide. A simulation of US infections predicts nearly 200,000 deaths associated with HCV infection and direct medical expenditures in excess of \$10 billion [2,3]. Current standards of care consist of peg-interferon- α (IFN α) and ribavirin, although this regimen is effective in only 50% of cases. In addition, this antiviral treatment is poorly tolerated and is associated with significant side effects and acquired resistance [4]. The recent release of HCV specific antivirals, such as Teleprevir (Vertex) and Boce-previr (Merck) offers to increase clearance rates to 70–80%, although incidents of HCV resistance are emerging. Therefore, there is a pressing need for the development of complementary and alternative treatment strategies to combat HCV infection, possibly through the targeting of host pathways on which the virus relies.

Recent work suggests a critical role for fatty acid and cholesterol metabolism in the HCV lifecycle. While the interaction between HCV infection and lipid metabolism initially received attention due to the development of steatosis [5], the lack of an efficient cell culture model of HCV limited research in the field. The development of a JFH1/Huh7.5 full lifecycle model of HCV infection enabled several groups to show that HCV replication is inhibited by statins [6,7]. This was shown to be in part due to the viral requirement for the geranylgeranylation of FBL2, a host factor which binds NS5A facilitating viral replication [6,8]. The replication of HCV on ER-associated lipid droplets has also been extensively characterized [9]. More recently, Gastaminza *et al.* demonstrated the existence of high density intracellular HCV precursors suggesting the virus binds to low density particles prior to egress [10]. Using a similar system, Huang *et al.* demonstrated that HCV assembled in vesicles enriched in ApoB and ApoE, structural proteins of very low-density lipoprotein (VLDL); and in microsomal triglyceride transfer protein (MTP), the rate-limiting enzyme in VLDL assembly [11]. Concomitantly, our group demonstrated that HCV is actively secreted while bound to VLDL [12,13], and that its production can be metabolically modulated by the addition of insulin and fatty acids. A comprehensive study of HCV effect on host metabolism was recently carried out [14].

As a consequence, modulators of hepatic lipid metabolism originally developed for atherosclerosis, could potentially interfere with HCV. Importantly, targeting a host pathway on which HCV relies, rather than a viral protein, may prove to be less susceptible to the emergence of resistant viral strains [15,16]. Despite the promise of such an approach, pilot *in vivo* trials using atorvastatin or bezafibrate failed to show significant effects on viral titer [17,18]. One compound that may prove efficient against HCV is the grapefruit flavonoid naringenin. Naringenin is a dietary supplement demonstrated to possess anti-oxidant, anti-inflammatory, and anti-carcinogenic properties both *in vitro* and *in vivo* [19]. A recent clinical trial in hypercholesterolemic patients demonstrated that naringenin's precursor, naringin, significantly lowered plasma LDL levels [20]. Similar cholesterol-lowering effects of naringenin were demonstrated in rabbits [21,22] and rats [23]. More recently, Huff and coworkers have shown that naringenin helps correct metabolic disturbances associated with diabetes in LDL receptor (LDLR) deficient mice [24]. These effects have been attributed to

MTP inhibition and thus potentially cause lipid accumulation and liver toxicity [25,26]. Interestingly, naringenin has also been shown to inhibit HMGCR, the rate-limiting enzyme in cholesterol synthesis, while activating enzymes important in fatty acid oxidation such as acyl-CoA oxidase (ACOX) and CYP450 4A1 [27], suggesting a transcriptional regulation of lipid metabolism, possibly on the nuclear receptor level.

In this work, we demonstrate that naringenin dose-dependently inhibits the secretion of ApoB and HCV particles, without affecting intracellular levels of viral RNA or protein. We show that naringenin prevents the accumulation of intracellular infectious particles, suggesting that the flavonoid blocks the assembly of infectious HCV particles. Importantly, PPAR inhibitor GW9662 reverses naringenin effects. We further show that in chronically infected cells, naringenin induces PPAR α , causing a decrease in triglyceride secretion without leading to hepatic lipid accumulation. Finally, we demonstrate that long-term treatment with naringenin leads to a rapid 1.4 log reduction in secreted HCV in cell culture and that this effect is reversible. Together, the data suggests that naringenin block the assembly of infectious HCV particles in cell culture and supports further investigation of naringenin in the management and care of HCV infection.

Materials and methods

Reagents

Lipoprotein-free FBS was purchased from Biomedical Technologies (Stoughton, MA). Naringenin, WY14, 643, GW9662, and Brefeldin A (BFA) were purchased from Sigma–Aldrich Chemicals (St. Louis, MO). Ciglitazone was purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals were purchased from Invitrogen Life Technologies (Carlsbad, CA) unless otherwise noted. BMS-200150, a small molecule inhibitor of MTP was provided by Pablo Gastaminza and Francis Chisari.

Cell culture and viruses

The Huh7.5.1 human hepatoma cell line and a plasmid containing the JFH-1 genome were kindly provided by Dr. Chisari (Scripps Research Institute, La Jolla, CA) and Dr. Wakita (National Institute of Infectious Diseases, Tokyo, Japan), respectively. Huh7.5.1 cells were cultured in DMEM supplemented with 10% FBS, 200 U/ml penicillin, and streptomycin in a 5% CO₂-humidified incubator at 37 °C. *In vitro* transcribed genomic JFH-1 RNA was delivered to cells by liposome-mediated transfection [28]. Subsequent rounds of infection were carried out using viral stocks produced by the cells. Infected Huh7.5.1 cells were passaged every 3 days and used at passage <15.

Hepatocyte isolation and culture

Primary human hepatocytes were obtained from BD Biosciences (San Jose, CA) or were kindly provided by Dr. Stephen C. Strom, University of Pittsburgh. Cells were plated on collagen-coated dishes at a density of 100,000 cells/cm² and cultured as previously described [29].

Cellular viability

Viability of primary human hepatocytes was quantified as previously described [30]. Briefly, medium samples were collected daily, frozen at –20 °C, and analyzed in bulk using Thermo Fisher Scientific (Waltham, MA) aspartate aminotransferase (AST) Infinity reagent. Values were normalized to the total amount of AST determined by total cell lysis [30]. Cell viability for all conditions was greater than 90%.

HCV Secretion

HCV-infected Huh7.5.1 cells were plated on a 6-well plate at a density of 2×10^5 cells/cm² and cultured overnight in the standard medium. Prior to the beginning of the experiment, the cells were washed 3 times with PBS and cultured with DMEM containing 5% lipoprotein-free FBS. Naringenin was added at this time as described in the text. Following 24 h of incubation, the plate was gently agitated to release mechanically bound particles, and the medium was collected, filtered to remove cellular debris, and stored at -80°C for further analysis. The attached cells were washed 3 times with PBS, harvested, pelleted, and stored at -80°C for further analysis.

Intracellular and extracellular HCV infectivity

The infectivity of HCV particles was measured as previously described [12]. Briefly, naïve Huh7.5.1 cells were grown to 80% confluence and exposed to cell culture supernatants serially diluted 10-fold in the culture medium. Following 1 h of incubation at 37°C , the medium was replaced, and cells were cultured for 3 additional days. Levels of HCV infection were determined by immunofluorescence staining for HCV core protein. The viral titer is expressed as focus forming units (FFU) per microliter of supernatant. To evaluate infectivity of intracellular particles, cells were scraped into PBS and lysed by freeze-thawing as previously described [13], diluted and used to assess infectivity as above.

GAL4-PPAR activation assays

PPAR activation was examined as previously described using the HGLN5 PPAR α and PPAR γ cell line [31]. Briefly, HGLN5 cells were seeded at a density of 100,000 cells/cm², test compounds were added 8 h later and incubated for 16 h. Following treatment, cells were washed with PBS and lysed in 25 mM Tris buffer (pH 7.8). Protein concentration was calculated using the Bradford assay and used to normalize the luciferase activity. Finally, activation of PPAR α and PPAR γ reporters is presented as percent of maximal activation by the known agonists GW7647 and BRL49653, respectively.

PPRE activation in JFH1-infected Huh7.5.1 cells

The pAOx(X2)luc plasmid which contains two tandem repeats of the AOX PPAR response element (PPRE) upstream of the firefly luciferase reporter gene [32] was a kind gift of Dr. John P. Capone (McMaster University, Canada). The pRL-TK plasmid containing the *Renilla* luciferase reporter under the control of a constitutive thymidine kinase promoter was purchased from Promega Corporation (Madison, WI). JFH1-infected Huh7.5.1 cells were transiently transfected with the pAOx(X2)luc and pRL-TK plasmids using Lipofectamine 2000 according to manufacturer's directions. Following 24 h incubation with naringenin, cells were lysed and assayed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. All measurements were done in triplicate and data are shown as values normalized to *Renilla* luciferase internal controls.

Human ApoB enzyme-linked immunosorbent assay (ELISA)

Huh7.5.1-secreted ApoB-100 was detected in the medium with the ALerCHEK, Inc. (Portland, ME), total human ApoB-100 ELISA kit. The medium was diluted 1:10 with the specimen diluent and the assay was carried out according to the manufacturer's directions.

HCV core ELISA

Huh7.5.1-secreted HCV core antigen was detected in the medium with the Wako Chemicals (Richmond, VA) ORTHO HCV antigen ELISA kit. The medium was used as is and the assay was carried out according to the manufacturer's directions.

MTP activity assay

MTP activity was measured using a commercial kit (Roar Biomedical, New York, NY). After 24 h stimulation of Huh7.5.1, cells were scraped into PBS on ice, centrifuged at 600g for 3 min and resuspended in buffer supplemented with protease inhibitor cocktail (Thermo Scientific). Cell suspensions were sonicated on ice. 100 µg cell lysates were combined with 10 µl of donor and acceptor particles in 220 µl assay buffer, and incubated at 37 °C. Increase in fluorescence was measured using SpectraMAX Gemini plate reader with excitation and emission of 465/538 nm. MTP activity was normalized to sample protein content.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Virus samples were purified using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). The reverse-transcription reaction step was performed on a Mastercycler egradientS (Eppendorf) instrument using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on a MyiQ Real-Time PCR Detection System using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA), according to the manufacturers' instructions. HCV detection primers used in qRT-PCR were Forward 5'-GGGAAGACTGGGTCCTTTCTTGGAT-3' and Reverse 5'-CGACGGTTGGTGTTCCTT TTGGTTT-3' (Integrated DNA Technologies, Coralville, IA) [12].

Lipid measurements

Intracellular triglycerides were quantified in primary hepatocyte cell extracts using a commercial kit (Sigma Chemical, St. Louis, MO) based on enzymatic hydrolysis of triglycerides by lipase to glycerol. Measurements were normalized to total protein content measured by the Bradford assay.

Long-term treatments

JFH1-infected Huh7.5.1 cells were grown over a course of 7 days in OptiMEM culture medium. Media was collected every 24 h and stored at -80 °C for analysis. On days 1-4 ("treatment"), media contained 200 µM naringenin, 1000 U/ml IFN α , or DMSO. On days 5-7 ("washout"), all wells were cultured in standard media without treatment. Primary human hepatocytes were similarly cultured for 9 days with media collected every 24 h and stored at -80 °C for analysis. Primary cells were treated days 1-7 with 200 µM naringenin, followed by days 8-9 washout period.

Statistics

Data are expressed as the mean \pm standard deviation. Unless noted differently, all experiments were carried out in triplicates. Statistical significance was determined by a one-tailed Student's *t* test. A *p* value of 0.05 was used for statistical significance.

Results

Naringenin inhibits the production of ApoB and HCV

Recently, our group and others demonstrated that HCV production is dependent VLDL secretion, and that naringenin blocked HCV production in JFH1-infected Huh7.5.1 cells [12]. To further characterize naringenin's antiviral activity we treated JFH1-infected Huh7.5.1 cells with increasing concentrations of naringenin for 24 h. Fig. 1A shows that naringenin led to a dose-dependent decrease in the secretion of ApoB and viral RNA, with an EC₅₀ of 109 µM. Maximal inhibition of secretion of both ApoB and HCV RNA was 74 \pm 4% at a concentration of 200 µM (*p* < 0.001). The secretion of HCV core protein was similarly decreased by 64 \pm 4% compared to untreated control (Fig. 1B). Naringenin causes

a similar inhibition in ApoB secretion in primary human hepatocytes (Fig. 4A). Interestingly, no change in intracellular HCV RNA abundance was noted up to 200 μM ($p = 0.43$) suggesting the compound does not dramatically affect viral replication. Some decrease in intracellular HCV RNA was noted at a concentration of 800 μM . Fig. 1B shows changes in HCV RNA and core protein, both intracellular and extracellular levels, compared to untreated controls following 24 h stimulation. Treatment did not lead to an intracellular accumulation of viral core protein or viral RNA (Fig. 1B).

Naringenin blocks the assembly of HCV infectious particles

We next wished to determine whether naringenin blocks the assembly of HCV prior to viral egress. To test this hypothesis, cells were treated with 0.1 $\mu\text{g/ml}$ brefeldin A (BFA), a toxin known to disrupt Golgi-dependent export [33]. BFA treatment was previously shown to cause the accumulation of intracellular infectious HCV particles in Huh7.5.1 infected cells [12,13]. Co-treatment of cells with BFA and the MTP-inhibitor, BMS-200150, blocks the accumulation of intracellular infectious HCV particles, suggesting MTP is required for HCV assembly [13]. As naringenin was previously shown to inhibit MTP activity, we explored if the compound similarly blocks HCV assembly.

JFH1-infected Huh7.5.1 cells were treated with 0.1 $\mu\text{g/ml}$ BFA and co-treated for 5 h with 10 μM BMS-200150 or 200 μM naringenin. The production of extracellular infectious particles (Fig. 1C) was significantly reduced when cells were treated with either BMS-200150 or naringenin (45% and 68%, respectively, $p < 0.02$) and abolished entirely when cells were treated with BFA ($p < 0.001$). Expectedly, BFA treatment which blocked the production of extracellular infectious particles, led to the accumulation of infectious HCV particles within the cells (Fig. 1D). Co-treatment with BMS-200150 decreased the accumulation of infectious particles by 46% ($p < 0.05$) compared to BFA-only controls. Importantly, co-treatment with naringenin decreased the accumulation of infectious particles by 55% ($p < 0.05$) compared to BFA-only controls (Fig. 1D) suggesting the flavonoid inhibits the assembly of HCV Lipo-viral particles [34].

Naringenin inhibits MTP activity while inducing PPAR activity

Naringenin has been previously shown to inhibit MTP, a critical enzyme in VLDL assembly, in HepG2 cells [25,26]. Previous reports have indicated that MTP inhibition can lead to lipid accumulation and steatosis. To test if naringenin leads to lipid accumulation, intracellular triglycerides were quantified in primary human hepatocytes following 24 or 96 h of treatment with 200 μM naringenin (Fig. 2A). Naringenin stimulation caused a decrease rather than an increase in intracellular triglyceride that was not judged to be statistically significant ($p = 0.1$ and 0.08, respectively).

Cellular lipid metabolism is controlled by nuclear receptors, members of a family of ligand-activated transcription factors [35]. Activation of PPAR α specifically, has been shown to induce β -oxidation and a reduction in lipogenesis and VLDL secretion [36]. Such activation could explain the lack of lipid accumulation. To examine if naringenin induces PPAR activation we used the HG5LN-PPAR3b1 reporter cell line [31]. In this cell line, the PPAR α ligand-binding domain (LBD) is fused to a GAL4 DNA binding domain. Activation of PPAR α -LBD allows GAL4 to bind its UAS response element transcribing luciferase. Fig. 2D shows that naringenin treatment enhanced the activity of the PPAR α -LBD by 4-folds ($p < 0.001$).

To examine whether the activation PPAR α -LBD in reporter cells correlates with PPRE activity in JFH1-infected Huh7.5.1 cells, we transfected cells with the pACOX(X2)luc reporter plasmid which contains two tandem repeats of the ACOX, PPAR response element

(PPRE) upstream of a firefly luciferase reporter [32]. *Renilla* luciferase expression was used as control. As shown in Fig. 2C, naringenin led to a dose-dependent increase in PPRE activity, reaching $23 \pm 7\%$ ($p = 0.015$) compared to DMSO-treated control. At $200 \mu\text{M}$ naringenin, PPRE induction was not statistically different from $10 \mu\text{M}$ of classical PPAR α agonist WY14643 ($p = 0.198$). Predictably, analysis of PPAR α and ACOX mRNA abundance by qRT-PCR demonstrated a 60% and 20% increase, respectively (Fig. 2E). Finally, PPAR α induction is known to inhibit cholesterol synthesis through SREBP and its target gene, *HMGR*. Fig. 2E shows that both transcripts are downregulated by naringenin.

To verify that PPAR α activation could indeed reduce ApoB and virus production in our system, we compared the treatment of $200 \mu\text{M}$ naringenin with $10 \mu\text{M}$ of WY14643 in Huh7.5.1 cells. Fig. 2B shows that both naringenin and WY14643 caused a significant $58 \pm 4\%$ and $42 \pm 5\%$ inhibition of MTP activity ($p < 0.002$), and $62 \pm 9\%$ and $33 \pm 13\%$ inhibition in ApoB secretion ($p < 0.02$). Similarly, both naringenin and WY14643 caused a significant $57 \pm 9\%$ and $63 \pm 7\%$ inhibition in HCV RNA secretion ($p < 0.012$), without affecting intracellular levels of HCV core protein at $97 \pm 11\%$ ($p = 0.70$) and $95 \pm 12\%$ ($p = 0.57$). Finally, the co-administration of naringenin with WY14643 did not increase PPRE activation ($p = 0.921$, $n = 3$) nor decrease APOB100 secretion ($p = 0.644$, $n = 3$), compared to naringenin treatment alone.

These results suggest that naringenin effect is mediated by PPAR α activation. We proceeded to explore if naringenin effects are reversed by PPAR α inhibition. Fig. 2F shows that $1 \mu\text{M}$ of PPAR antagonist GW9662 inhibited PPRE activation in the presence of $200 \mu\text{M}$ naringenin by 33% ($p < 0.001$) and increased ApoB secretion by 78% ($p < 0.001$). GW9662 also increased extracellular infectivity by 34% ($p < 0.05$) while the accumulation of intracellular infectious viral particles increased by 50% ($p < 0.05$). These results strongly suggest that naringenin's effects are at least partially mediated by PPAR α activation.

Long-term inhibition of HCV production

To explore the long-term effects of naringenin treatment on viral production we treated chronically infected Huh7.5.1 cells with $200 \mu\text{M}$ naringenin over a course of 4 days (treatment). As a positive control, cells were treated with 1000 i.u. of IFN α , a concentration previously shown to abolish viral production. On days 5–7, treatment was removed from all cells (washout).

As expected, our results showed that ApoB secretion was decreased by $86 \pm 1\%$ following naringenin treatment ($p < 0.01$), but not by IFN α that showed a $16 \pm 3\%$ increase (Fig. 3). Importantly, naringenin treatment caused a rapid 1.4 log reduction in HCV RNA and a $67 \pm 8\%$ reduction in HCV core ($p < 0.05$). This inhibition in HCV production was similar to IFN α , which showed a maximal 1.7 log reduction in HCV RNA and an 83% reduction in HCV Core (Fig. 3). Intracellular levels of HCV RNA remained unchanged through the course of naringenin treatment (Supplementary Fig. 1). Not surprisingly, during the washout period, HCV levels in naringenin-treated cells returned to normal suggesting that the compound did not affect viral replication.

Naringenin effects in primary human hepatocytes

The metabolic activity and gene expression of hepatoma cell lines, like Huh7.5.1, are significantly lower than those of primary hepatocytes. To test whether naringenin effects translate to a more relevant model of liver metabolism, we examined its activity in stabilized cultures of primary human hepatocytes as previously described [30]. Fig. 4A shows that naringenin caused a dose-dependent inhibition of ApoB secretion in primary human hepatocytes, reaching $38 \pm 14\%$ inhibition at $400 \mu\text{M}$ concentration. Similarly, Fig. 4B

shows that PPRE activation increased by 3-fold following 200 and 400 μM naringenin stimulation, not significantly different than 10 μM WY14643 ($p = 0.129$). Finally, Fig. 4C demonstrates the long-term transient inhibition of ApoB secretion in primary human hepatocytes. Following 7 days of continuous treatment with 200 μM naringenin, ApoB secretion decreased by $43 \pm 8\%$ ($p < 0.01$) while cell viability remained unchanged ($p = 0.137$). During the washout period, ApoB secretion returned to $86 \pm 15\%$ of basal level ($p = 0.174$). Intracellular triglyceride levels remained unchanged following 1 or 4 days of continuous treatment (Fig. 2A).

Discussion

HCV is a global public health problem affecting close to 3% of the world population. The current standard of care consists of peg-IFN α and ribavirin, effective in approximately half of the patients, and associated with the emergence of resistant strains [4,37]. While protease and polymerase inhibitors developed the context of STAT-C are showing remarkable potential, indications of resistant strains have already been reported in clinical trials [16]. A complimentary approach is to target a host pathway, such as VLDL assembly, on which the virus depends. HCV would require numerous mutations and a significant alteration of its lifecycle to escape, essentially becoming a different virus.

Targeting host metabolic pathways on which HCV relies could deprive the virus from critical resources needed to complete its lifecycle. Such strategy may prove to be less susceptible to the emergence of resistant viral strains [15,16]. Despite the promise of such an approach, pilot *in vivo* trials using atorvastatin or bezafibrate failed to show significant effects on viral titer [17,18]. The concentration of atorvastatin needed to effect viral replication was judged to be too close to its toxic dose, while 8 weeks of treatment with PPAR α agonist bezafibrate lowered viral titer by only 30% [17,18]. It is hoped that another agent would be more successful is blocking the relevant pathways.

Our group has previously suggested that the grapefruit flavonoid naringenin could be effective in inhibiting HCV production, although its mechanism of action was unclear. At the time, naringenin was considered an MTP inhibitor, thus likely to cause lipid accumulation and liver toxicity [12]. Interestingly, the compound demonstrates anti-inflammatory and normolipidemic effects *in vitro* and *in vivo* [19]. The ability of naringenin to significantly reduce plasma cholesterol levels has been demonstrated in a recent clinical trial in hypercholesterolemic patients in which the compound lowered LDL levels by 17% [20]. Similar cholesterol lowering effects were demonstrated in rabbits [21,22] and rats [23]. Another appealing aspect of using naringenin, is that the compound has demonstrated anti-cancer properties, blocking *in vivo* replication of HepG2, Hep3B, and Huh7 cells [38]. As HCV dramatically increases the risk of liver cancer, inhibiting tumor formation is especially important.

In this study, we first showed that naringenin induced a dose-dependent inhibition of HCV RNA and core protein secretion, down to 28% of untreated controls (Fig. 1). The reduction in viral RNA and protein production correlated with inhibition of HCV infectivity. On the other hand, intracellular HCV RNA and protein levels showed no change in response to naringenin treatment, suggesting naringenin was inhibiting a step downstream of viral replication. Nonetheless, given the reduction in viral secretion, the lack of intracellular viral RNA and protein accumulation may suggest a concurrent inhibition of viral replication or enhanced viral degradation.

Assembly of infectious HCV particles has been previously demonstrated to be dependent on MTP activity, an enzyme critical to VLDL assembly. The accumulation of infectious viral

particles in cells treated with BFA, a toxin that disrupts Golgi-mediated transport, is consistent with the observations of Chisari and coworkers [12,13]. Here we showed that naringenin, like MTP inhibitor BMS-200150, blocks the accumulation of infectious particles in BFA-treated cells up to 55% ($p < 0.05$) suggesting that the flavonoid blocks the assembly of infectious viral particles. Consistent with this observation, we showed that naringenin inhibited MTP activity, consistent with prior observations [25]. In a recent clinical study, Cuchel and colleagues showed that while the MTP inhibitor BMS-201038 (AEGR-733) lowered LDL values it was associated with steatosis and elevated ALT levels, leading to an early study termination [39]. To test if naringenin leads to a similar lipid accumulation, intracellular triglycerides were quantified in primary human hepatocytes following 24 or 96 h of treatment with 200 μM naringenin. Remarkably, there was no lipid accumulation in naringenin treated cells.

The ability of naringenin to modulate the expression of genes important in fatty acid oxidation, led us to examine its effects on PPAR α . We demonstrate that naringenin specifically activates the ligand-binding domain of PPAR α (Fig. 2). Consistently, naringenin showed a dose-dependent increase in PPRE activity in Huh7.5.1 cells and primary human hepatocytes, similar to PPAR α classical agonist WY16463. Gene expression studies also correlate with a PPAR α -induced shift from cholesterol synthesis to fatty acid oxidation. The inhibition of HMGCR could deprive the virus from associating with geranylgeranylated FBL-2 inhibiting its replication, potentially explaining the decrease in HCV RNA at high naringenin concentration (Fig. 1A). However, this does not appear to be naringenin's main mechanism of action. Importantly, JFH1-infected Huh7.5.1 cells treated with WY16463, show a similar inhibition of MTP activity, ApoB and HCV RNA secretion to naringenin. Like naringenin, WY 16463, did not affect intracellular levels of HCV core protein (Fig. 2B). Finally, to demonstrate that naringenin effects are mediated by PPAR α activation, we explored its activity in the presence of PPAR α and PPAR γ irreversible antagonist GW9662. We showed that in the presence of GW9662, naringenin effects were reversed with PPRE activity reduced, while ApoB secretion, HCV secretion, and HCV assembly increased by 78%, 34%, and 50% (Fig. 2F). These results strongly suggest that naringenin's effects are at least partially mediated by PPAR α activation. Interestingly, chronic HCV infection has long been associated with steatosis [40,41], possibly through the inhibition of PPAR α by viral proteins [42,43]. One intriguing possibility is that PPAR α activation by compounds such as naringenin could reduce steatosis and its effects by augmenting hepatic fatty acid oxidation.

Finally, we demonstrate that naringenin inhibits long-term virus production and that its effects are reversible both in chronically infected Huh7.5.1 cells and primary human hepatocytes. As expected, naringenin reduced ApoB secretion by 86%. This effect was not observed in IFN α -treated cells. When media was examined for viral core and RNA content, both naringenin and IFN α significantly reduced the secretion of HCV RNA by 1.4 and 1.7 log, respectively. Importantly, naringenin's effect on viral secretion was reversible, as viral secretion rebounded during washout, consistent with our proposed mechanism of action. We note that the decrease in core protein secretion on day 5, was not statistically significant ($p = 0.214$), and considered to be an artifact of normalization.

Our results suggest that naringenin blocks the assembly of infectious HCV particles. The tight link between HCV and VLDL assembly, in which infectious virus takes the form of lipo-viral particles, suggests a new approach for combating the virus. We expect that targeting host metabolism rather than viral proteins will decrease the development of resistant strains. As HCV does not integrate into the host DNA and relies on continuous infection of fresh hepatocytes to persist, blocking its ability to produce infectious particles could dramatically affect its persistence. Moreover, application of naringenin or other PPAR α agonists with STAT-C agents could bring a rapid reduction in HCV levels [44],

especially during the early treatment phase. Such early rapid reduction in HCV levels has been associated with sustained virological response [4]. Our work strongly supports further investigation of naringenin in the management of HCV infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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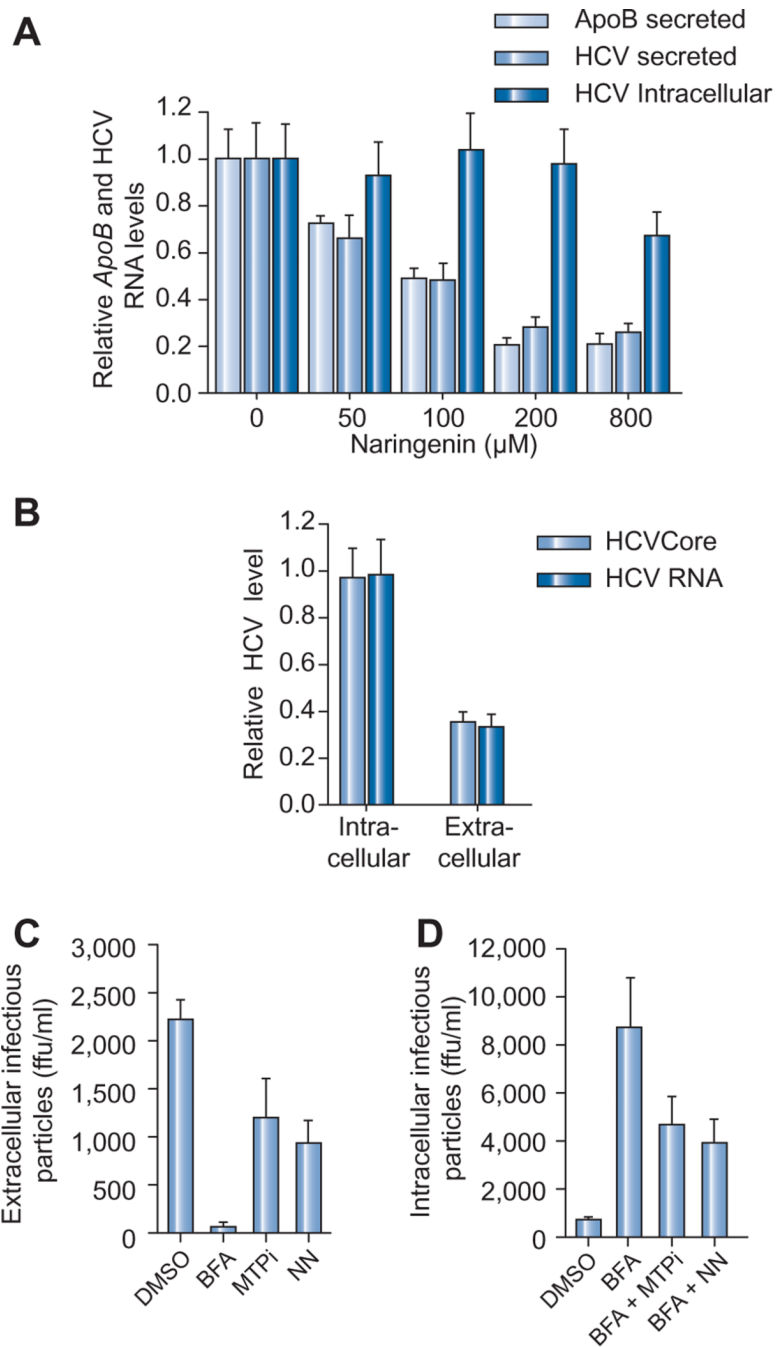


Fig. 1. Naringenin blocks the assembly of HCV-infectious particles

(A) Naringenin dose-dependently inhibits ApoB-100 and HCV RNA secretion in JFH1-infected Huh7.5.1 cells. HCV RNA did not accumulate in cells. Maximal inhibition of HCV RNA secretion was $74 \pm 4\%$ at $200 \mu\text{M}$ naringenin at 24 h ($p < 0.001$). (B) 24 h of treatment with $200 \mu\text{M}$ naringenin decreased the secretion of virus into the media, but did not change the amount of viral RNA or core protein inside cells. Values are normalized to untreated controls. (C) Infected cells were treated for 5 h with $0.1 \mu\text{g/ml}$ BFA, an inhibitor of Golgi-dependent secretion, $10 \mu\text{M}$ of BMS-200150, a known MTP inhibitor (MTPi), or with $200 \mu\text{M}$ naringenin (NN). Infectivity of the secreted virus (extracellular) was quantified by colony titer. Extracellular infectivity was blocked by BFA treatment and inhibited by both

BMS-200150 and naringenin, by 45% and 68%, respectively ($p < 0.02$). (D) To measure changes in the accumulation of intracellular infectious virus particles, the cells were lysed by freeze–thaw cycle and the virus-containing supernatant was used in an infectivity titer assay. BFA treatment led to accumulation of infectious virus in cells. Co-treatment of BFA with BMS-200150 blocked the accumulation of infectious virus in cells, leading to a 46% ($p < 0.05$) decrease in infectious particle accumulation. Naringenin had a similar effect to that of the MTP inhibitor, leading to a 55% ($p < 0.05$) decrease in infectious particle accumulation.

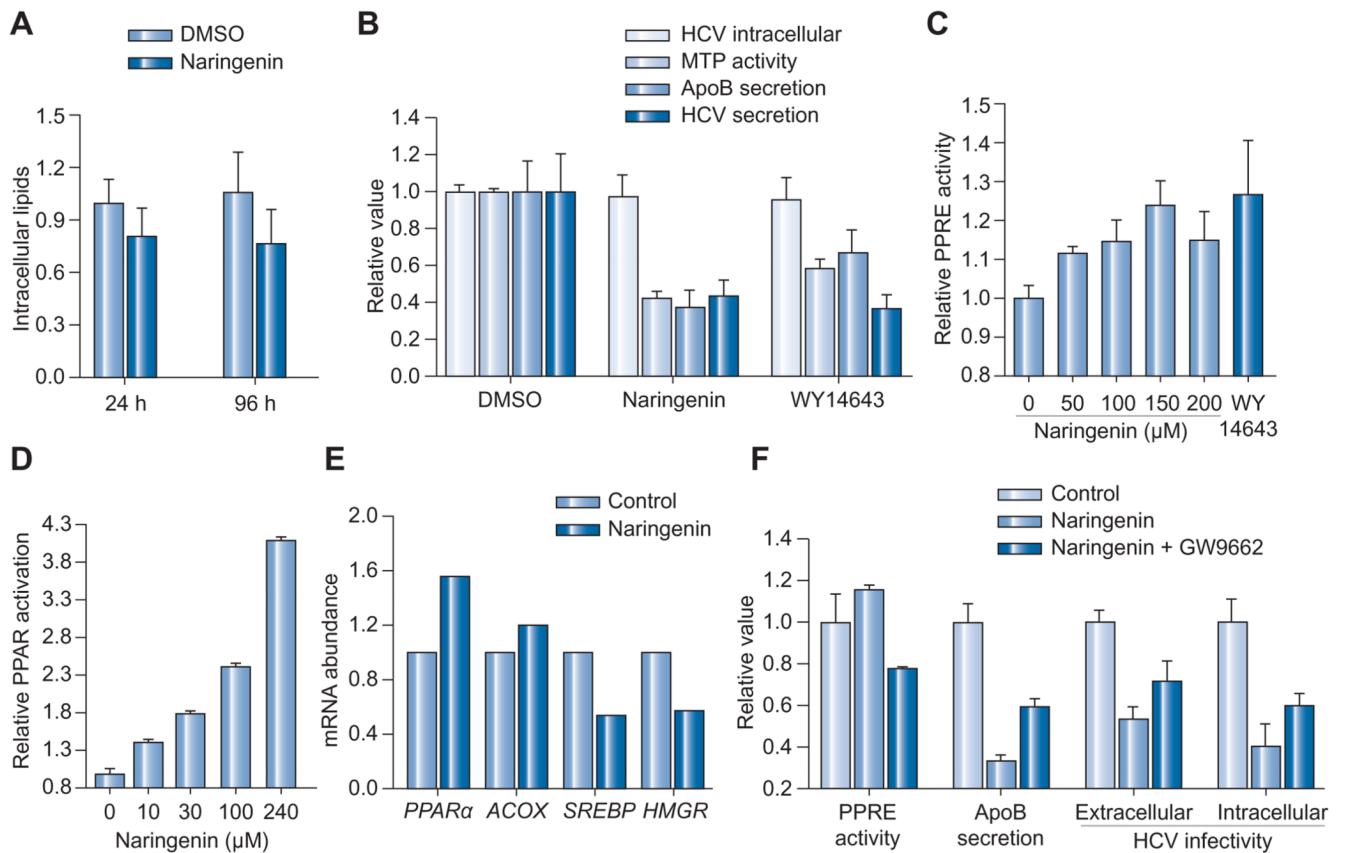


Fig. 2. The activity of naringenin is mediated in part by PPAR α

(A) Continuous 24 or 96-h treatment with 200 μ M naringenin does not cause triglyceride accumulation in primary human hepatocytes. (B) JFH1-infected Huh7.5.1 cells treated with 200 μ M naringenin or 10 μ M WY14643, a PPAR α agonist, show a similar inhibition of MTP activity ($p < 0.002$), ApoB secretion ($p < 0.02$), and HCV RNA secretion ($p < 0.012$), without affecting intracellular levels of HCV core protein ($p > 0.50$). (C) Chronically infected Huh7.5.1 cells transfected with PPRE reporter plasmid were treated for 24 h with naringenin. Naringenin dose-dependently enhanced PPRE activity by up to $24 \pm 7\%$ ($p = 0.015$). Maximal induction was not different from WY14643 ($p = 0.198$). (D) HG5LN-PPAR α reporter cells in which the PPAR α LBD is fused to GAL4 were treated for 16 h with naringenin. Naringenin enhanced PPAR α -LBD activity by $140 \pm 5\%$ ($p < 0.001$). (E) qRT-PCR analysis of JFH1-infected Huh7.5.1 cells treated with 200 μ M naringenin for 24 h. Naringenin caused increased expression of PPAR α and its target gene ACOX and a concomitant decrease in SREBP and its target HMGR. (F) 1 μ M of PPAR antagonist GW9662 inhibited PPRE activity in the presence of 150 μ M naringenin by 30% ($p < 0.001$) and increased ApoB secretion by 78% ($p < 0.001$). Extracellular HCV infectivity was increased by 34% ($p < 0.05$) while the accumulation of intracellular infectious HCV particles in the presence of 0.1 μ g/ml BFA increased by 50% ($p < 0.05$).

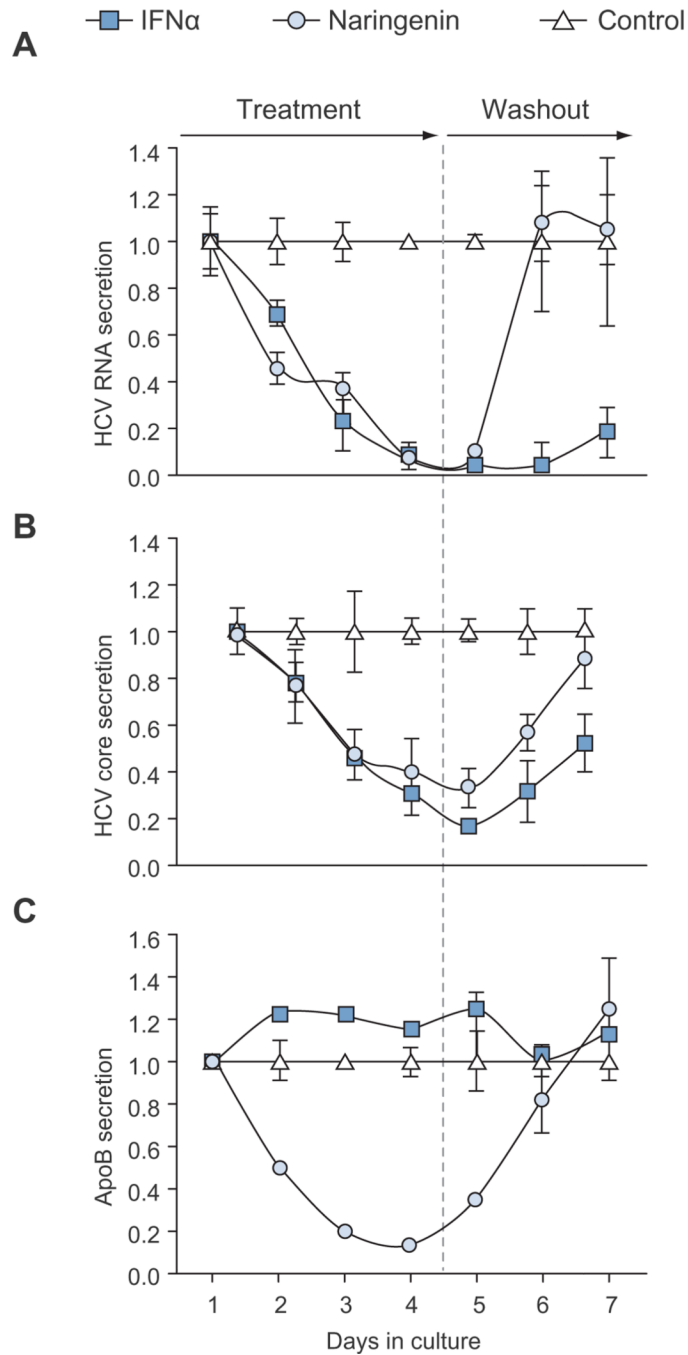


Fig. 3. Naringenin causes a rapid inhibition of HCV production during long-term treatment
 Cells were treated daily for 4 days with 200 μ M naringenin or 1000 i.u. IFN α . During the 3 days washout period, all wells were untreated. (A) Viral RNA release into media decreased during treatment, down to 4% with naringenin and 2% with IFN α compared to controls. During washout phase, naringenin-treated samples contained RNA levels similar to controls, suggesting viral replication was unchanged. (B) HCV core levels in media decreased during treatment for naringenin and IFN α . For naringenin-treated cells on days 4, core in the media was reduced to $33 \pm 5\%$. Core accumulation was partly restored during the last day of washout in both naringenin and IFN α -treated samples $87 \pm 12\%$, $52 \pm 13\%$, respectively. (C)

ApoB secretion declined during treatment with naringenin. On days 4, ApoB secretion in naringenin-treated cells decreased to $13 \pm 1\%$.

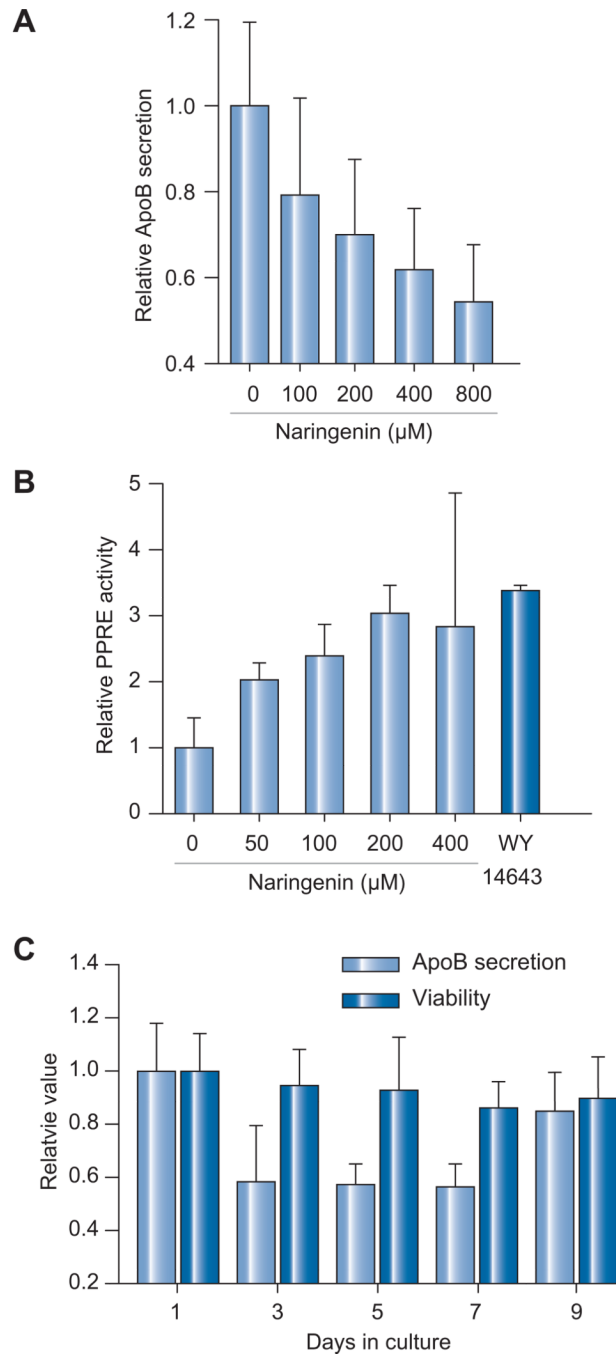


Fig. 4. Naringenin effects on primary human hepatocytes

(A) Naringenin caused a dose-dependent inhibition in ApoB-100 secretion following 24 h of treatment of primary human hepatocytes. Cell viability was >95%. (B) Primary human hepatocytes transfected with PPRE reporter plasmid were treated with naringenin. Naringenin dose-dependently enhanced PPRE activity following 24 h of treatment by 3-fold following 200 and 400 μM naringenin stimulation, not significantly different than 10 μM WY14643 ($p = 0.129$). (C) Primary human hepatocytes were treated for 7 days with 200 μM naringenin followed by a 2 day washout period. ApoB secretion decreased by $43 \pm 8\%$ ($p < 0.01$) while cell viability remained unchanged ($p = 0.137$). During the washout period, ApoB secretion returned to $86 \pm 15\%$ of basal level ($p = 0.174$).