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Divergent antiviral effects of bioflavonoids on the hepatitis C virus life cycle ☆

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ABSTRACT

We have previously demonstrated that quercetin, a bioflavonoid, blocks hepatitis C virus (HCV) proliferation by inhibiting NS5A-driven internal ribosomal entry site (IRES)-mediated translation of the viral genome. Here, we investigate the mechanisms of antiviral activity of quercetin and six additional bioflavonoids. We demonstrate that catechin, naringenin, and quercetin possess significant antiviral activity, with no associated cytotoxicity. Infectious virion secretion was not significantly altered by these bioflavonoids. Catechin and naringenin demonstrated stronger inhibition of infectious virion assembly compared to quercetin. Quercetin markedly blocked viral translation whereas catechin and naringenin demonstrated mild activity. Similarly quercetin completely blocked NS5A-augmented IRES-mediated translation in an IRES reporter assay, whereas catechin and naringenin had only a mild effect. Moreover, quercetin differentially inhibited HSP70 induction compared to catechin and naringenin. Thus, the antiviral activity of these bioflavonoids is mediated through different mechanisms. Therefore combination of these bioflavonoids may act synergistically against HCV.

Introduction

The hepatitis C virus (HCV) infects 3% of the world population and is mainly responsible for liver transplantation in patients with cirrhosis in developed countries (Shepard et al., 2005). Furthermore, HCV is the most common chronic blood borne pathogen in the United States (US) affecting 1.8% of the population and is the major etiologic factor responsible for the recent doubling of hepatocellular carcinoma (HCC) (El-Serag, 2002).

HCV infection is currently treated by pegylated interferon-α (PEG-IFN) and ribavirin with a sustained virologic response (SVR) of only 50–56% in patients with genotype 1 (Gambarin-Gelwan and Jacobson, 2008). Adverse side effects and contraindications are common for therapy of all genotypes as well (McHutchison and Patel, 2002). Recently, NS3/4A protease inhibitors in combination with PEG-IFN and ribavirin led to an increased SVR, but also increased adverse events including anemia and gastrointestinal symptoms (Ciesek and Manns, 2011). Nevertheless, a significant number of patients cannot receive these treatments as they require PEG-IFN. For these reasons, there is the need to develop adjunct or replacement therapies that are less toxic and more efficacious in terms of higher SVR rates and HCC prevention.

The 5′ non-coding region (NCR) of the viral genome possesses an internal ribosomal entry site (IRES) (Wang et al., 1993), a *cis*-acting

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; PEG-IFN, pegylated interferon-α; SVR, sustained virological response; NCR, noncoding region; IRES, internal ribosomal entry site; NS, nonstructural; NS5A, nonstructural protein 5A; HSP, heat shock protein; GFP, green fluorescent protein; RLuc, *Renilla* luciferase; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; HCVcc, HCV cell culture; ORF, open reading frame; FLuc, *Firefly* luciferase

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element found in some host RNA transcripts as well as in viruses that allows ribosomal translation initiation to occur internally within a transcript in lieu of 5′-cap dependent translation (Pacheco and Martinez-Salas, 2010). The HCV viral life cycle in a cell can be divided into six phases: (1) binding and internalization, (2) cytoplasmic release and uncoating, (3) viral polyprotein translation and processing, (4) RNA genome replication, (5) packaging and assembly, and (6) virus morphogenesis and secretion (Moradpour et al., 2007).

The viral nonstructural protein 5A (NS5A), a 56–59 kDa phosphoprotein, is a multi-functional protein and a component of the viral replicase complex. It has been implicated in regulation of HCV genome replication, viral protein translation, virion assembly, and viral secretion (He et al., 2003; Hughes et al., 2009; Tellinghuisen et al., 2008). We have previously identified, through co-immunoprecipitation, an NS5A/HSP complex composed of NS5A, HSP70, and HSP40 (cofactor of HSP70) and demonstrated their colocalization in Huh-7 cells (Gonzalez et al., 2009). We further showed that both NS5A-augmented IRES-mediated translation and virus production are blocked by (1) HSP70 knockdown, (2) the HSP synthesis inhibitor quercetin, a bioflavonoid, and (3) a small peptide from NS5A domain I that is capable of blocking NS5A/HSP70 interaction, with no associated cytotoxicity (Gonzalez et al., 2009; Khachatoorian et al., 2012). These findings support our hypothesis that HCV utilizes an NS5A/HSP complex to facilitate IRES-mediated translation of its genome and that disruption of this complex by quercetin-mediated knockdown of HSP70 blocks viral proliferation, implicating quercetin as a potential HCV treatment option.

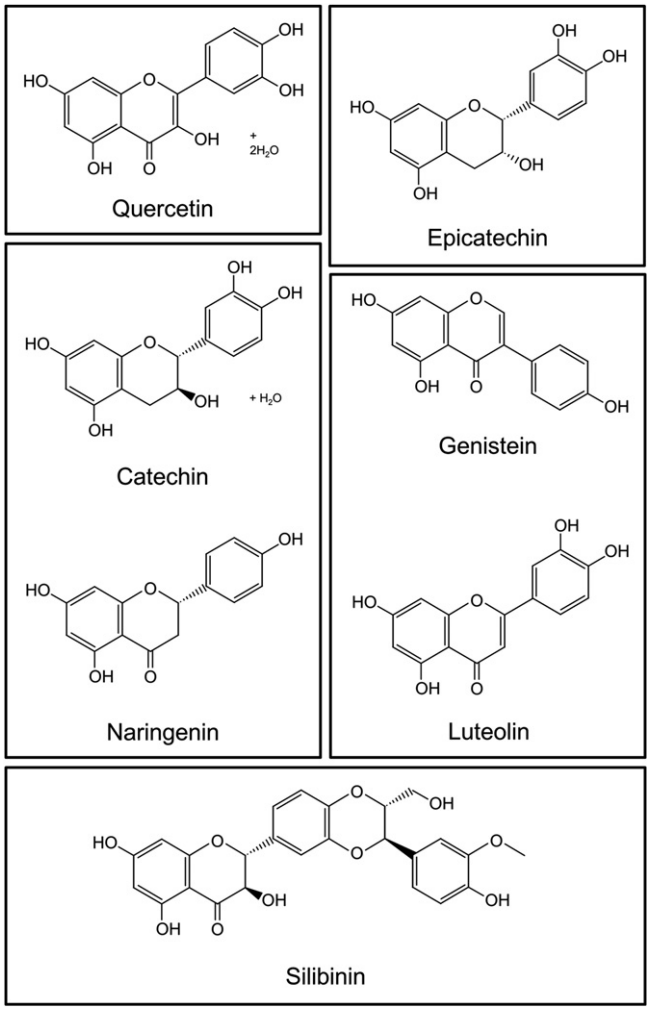

Fig. 1. Molecular structure of the bioflavonoids. Bioflavonoid molecular structures are adapted from Sigma-Aldrich. For naringenin, the structure of the (S) enantiomer is shown; however, the chemical is a racemic mixture of both enantiomers. Silibinin is the major active component of silymarin.

Bioflavonoids are a group of plant secondary metabolites that serve a variety of functions in plants including pigmentation and resistance to predators. The basic structure of bioflavonoids consists of two phenyl moieties linked together by three carbons (Fig. 1A). A variety of functional groups occur at different positions on this backbone to give rise to the large selection of these naturally occurring compounds. In recent years, bioflavonoids have been extensively studied for their health benefits.

In this study, we sought to examine, in more detail, the effect of quercetin and a number of bioflavonoids structurally related to quercetin on viral proliferation and to obtain further insights into the mechanisms of bioflavonoid-mediated suppression of HCV production.

Results

We have previously shown that quercetin blocks virus production with no associated cytotoxicity (Gonzalez et al., 2009). Here, we sought to determine if a number of other bioflavonoids structurally related to quercetin would also possess antiviral activity. We tested a total of seven compounds from a variety of bioflavonoid groups: catechin and epicatechin (flavanols), genistein (an isoflavone), luteolin (a flavone), naringenin (a flavanone), quercetin (a flavonol), and silymarin (a mixture of flavonolignans, where silibinin is the major active component (Wellington and Jarvis, 2001)) (Fig. 1).

Screening of bioflavonoids for cellular toxicity

We first determined the cellular toxicity of these seven compounds at a concentration range of 25 and 125 μM using standard MTT assays. As shown in Fig. 2A, the bioflavonoids can be divided into three groups based on their cytotoxicity profiles. Genistein and luteolin, potential chemotherapeutic agents, display significant cytotoxicity and were not studied further. Naringenin, quercetin, and silymarin possess a cytotoxicity profile similar to DMSO carrier. Interestingly, catechin and epicatechin result in significantly increased absorbance. This may reflect an increase in metabolism, cell division, or cell size; decreased apoptosis; or increased mitochondrial biogenesis. This is a subject of ongoing study. In a separate experiment, we analyzed the toxicity of all bioflavonoids in a time course of 72 h, and we obtained a similar toxicity profile (Fig. 2B).

Catechin, quercetin, and naringenin significantly attenuate HCV production in a dose-dependent manner

Next, we tested the bioflavonoids for their antiviral activity using the HCV cell culture (HCVcc) system measuring intracellular levels of HCV-driven protein expression. Based on our preliminary analyses, we found the concentration range of 25–125 μM bioflavonoids to be the most informative for the assays conducted in this study in terms of distinguishing bioflavonoid effects on the stages of viral life cycle. Initially, huh-7.5 cells were infected with the reporter virus and treated with 25 μM bioflavonoid for 48 h, followed by measuring luciferase activity. Quercetin and naringenin displayed significant antiviral activity in agreement with previous reports (Goldwasser et al., 2011; Gonzalez et al., 2009) (Fig. 3A). In addition, we found catechin to possess significant antiviral activity as well (Fig. 3A).

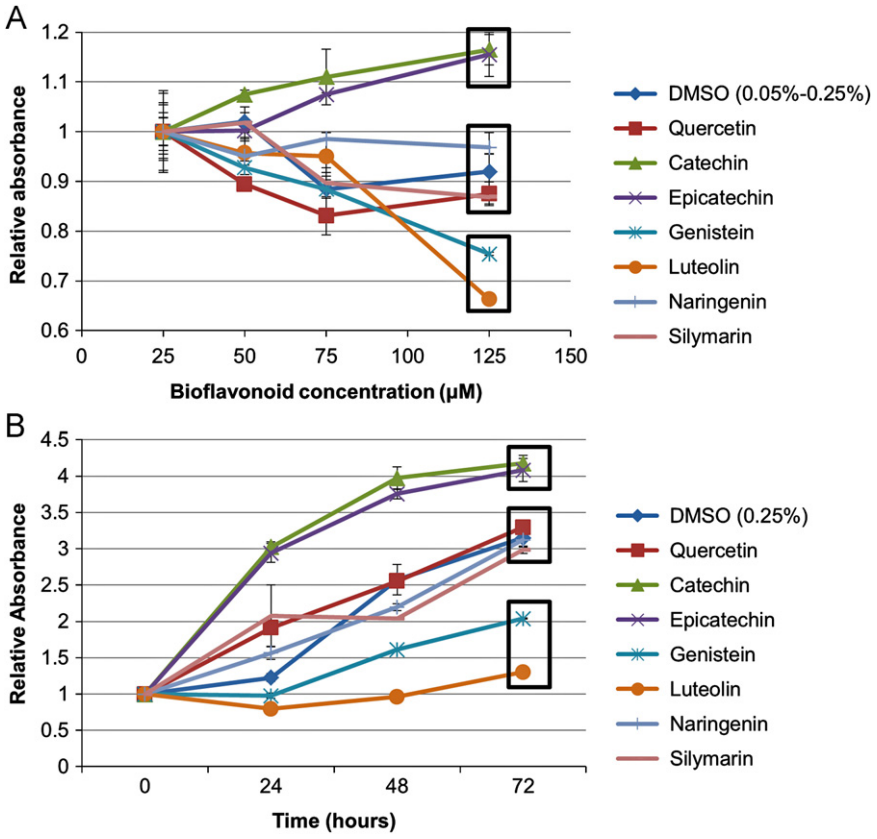

Fig. 2. Cytotoxicity of bioflavonoids as determined by MTT assays. (A) Bioflavonoid cellular toxicity profile in a concentration range of 25–125 μM. MTT assays were performed 72 h post treatment. (B) Bioflavonoid cellular toxicity profile in a time course of 72 h at 125 μM. All data was normalized to time 0. Boxes indicate flavonoids grouped together based on similar toxicity profiles. (Error bars reflect standard deviation.)

Interestingly, epicatechin, a diastereoisomer of catechin, displayed a slight increase in virus production (Fig. 3A). We are currently investigating the opposite effects of catechin and epicatechin on viral proliferation levels. The observed antiviral activities were not indirect effects of bioflavonoids on luciferase expression as determined independently by transfection of a plasmid expressing *Renilla* luciferase (Fig. 3B). Silymarin did not result in viral attenuation at 25 μM dose (Fig. 3A). None of the compounds displayed any cytotoxicity in MTT assays (Fig. 3C). We speculated that higher concentrations of silymarin may display some antiviral activity as reported previously (Wagoner et al., 2010). As shown in Fig. 3D, 125 μM silymarin resulted in a modest antiviral activity in a 48 h assay. However, silymarin was not studied further due to its significantly lower antiviral activity compared with the other bioflavonoids.

Intracellular viral levels were also determined with a treatment range of 25–125 μM catechin, naringenin, and quercetin for 72 h. All three compounds displayed a dose-dependent antiviral activity with quercetin being the most potent bioflavonoid followed by catechin and naringenin (Fig. 4A). To further confirm the antiviral activity of these compounds, the supernatants of these cultures were concentrated 30-fold to remove approximately 97% of bioflavonoids, and the concentrated supernatants were used to infect naïve cells. As shown in Fig. 4B, infectious virus production was significantly decreased in a dose-dependent manner.

To further confirm the antiviral activity of catechin, naringenin, and quercetin, huh-7.5 cells were infected and treated with catechin, naringenin, and quercetin for 72 h as above, followed by measuring viral RNA levels by quantitative reverse transcriptase PCR as well as assaying NS5A protein levels by Western analysis. As shown in Fig. 4C and D, all three compounds significantly reduced viral RNA and NS5A protein levels, in agreement with the luciferase reporter levels.

Quercetin markedly inhibits intracellular viral protein production compared to catechin and naringenin

We proceeded to determine the mechanism(s) of action of catechin, quercetin, and naringenin, using the HCVcc system. To test the bioflavonoid inhibition of viral protein production, huh-7.5 cells were infected and immediately treated with 125 μM of bioflavonoid. 125 μM concentration was chosen as it was optimal for distinguishing the mechanism of antiviral activity of the bioflavonoids. Luciferase assays 20 h after treatment showed that catechin, quercetin, and naringenin significantly inhibited intracellular viral protein translation, with quercetin demonstrating more than two-fold higher activity than catechin and naringenin (Fig. 5A). The 20-h assay time is crucial to limit the assay to one viral life cycle (see below) and eliminate any possible effects of bioflavonoids on other stages of viral life cycle such as virion assembly and secretion.

We also monitored translation levels within 28 h after infection. As shown in Fig. 5B, with no bioflavonoid treatment, viral translation levels steadily increased followed by sharp burst in viral translation between 24 and 28 h time points. This was interpreted to result from secondary infection. From this we inferred that the viral cycle duration is between 20 and 24 h. Bioflavonoid treatment significantly decreased viral protein production during one viral life cycle and afterwards (Fig. 5B). Quite interestingly, quercetin, our most potent bioflavonoid, inhibits

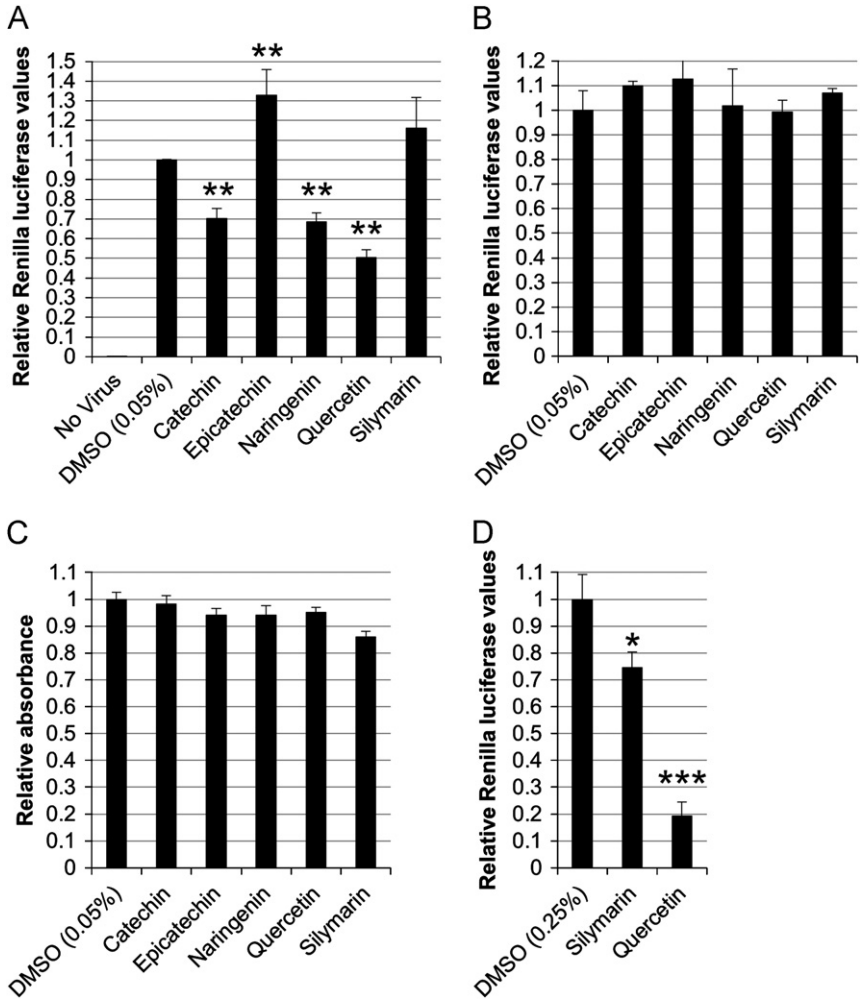

Fig. 3. Bioflavonoid antiviral activity. (**A**) Effect of 25 μM bioflavonoids on viral proliferation. Huh-7.5 cells were infected with the reporter virus and immediately treated with bioflavonoids. Forty-eight hours post treatment, luciferase activity was assayed. (B) Effect of bioflavonoids on *Renilla* activity. Luciferase assays were performed on cells transfected with pRL-TK plasmid. (C) MTT assays performed in parallel with assays in parts (A) and (B). (D) Effect of 125 μM silymarin on viral proliferation. Huh-7.5 cells were infected with the reporter virus and treated with bioflavonoids for 48 h as in part (A), followed by luciferase assay. (*, **, and *** indicate $P < 0.05$, $P < 0.005$, and $P < 0.0005$, respectively. Error bars reflect standard deviation.)

any increase in viral protein levels during and after one viral life cycle (Fig. 5B).

Infectious virion secretion is not inhibited by catechin, naringenin, and quercetin

To determine the effect of bioflavonoids on infectious virion secretion, huh-7.5 cells were infected with the reporter virus. After 24 h, allowing for sufficient accumulation of intracellular virus, cells were washed to remove secreted virions and treated with 125 μM bioflavonoid for 5 h. This limited time of treatment is necessary to minimize their effect on viral protein production. Subsequently, supernatants were concentrated 30-fold to remove approximately 97% of bioflavonoids. Elimination of bioflavonoids is necessary to exclude their potential subsequent effects on infection of naïve cells. The concentrated supernatants were used to infect naïve cells. While catechin and quercetin slightly affected viral secretion compared with DMSO, the differences were not statistically significant and may reflect their potent translation inhibitory effects during the 5 h infection period (Fig. 6A). As a positive control, treatment with Brefeldin A (BFA), an inhibitor of Golgi-dependent secretion including viral secretion (Goldwasser et al., 2011), did significantly reduce viral secretion (Fig. 6A).

We also ensured that concentrating the culture supernatants indeed removes the bioflavonoids. Aliquots of culture medium were prepared containing 125 μM of catechin, naringenin, or quercetin. Two sets of huh-7.5 cells were infected. After the infection period, the supernatants of one set of cells were replaced by the media containing the bioflavonoids as done for all assays in this study. However, for the second set of cells, the medium was subjected to filtration in the same manner as above, and the cleared medium was used to replace the culture supernatants. Luciferase activity was assayed for both sets 48 h after infection. The filtration step abolished the antiviral activity that was observed with no filtration (Fig. 6B).

Catechin, naringenin, and quercetin significantly inhibit intracellular infectious virion assembly

We next determined the effect of bioflavonoids on viral assembly. Two sets of huh-7.5 cells were infected and treated with BFA. One set was used for the assembly assay and the 'control set' for measuring viral translation (below). BFA treatment was performed to trap all assembled virions in the cytoplasm of infected cells. This step is necessary to limit the effect of bioflavonoid treatment to viral assembly alone, as opposed to viral secretion. Three hours later, cells were treated with 125 μM

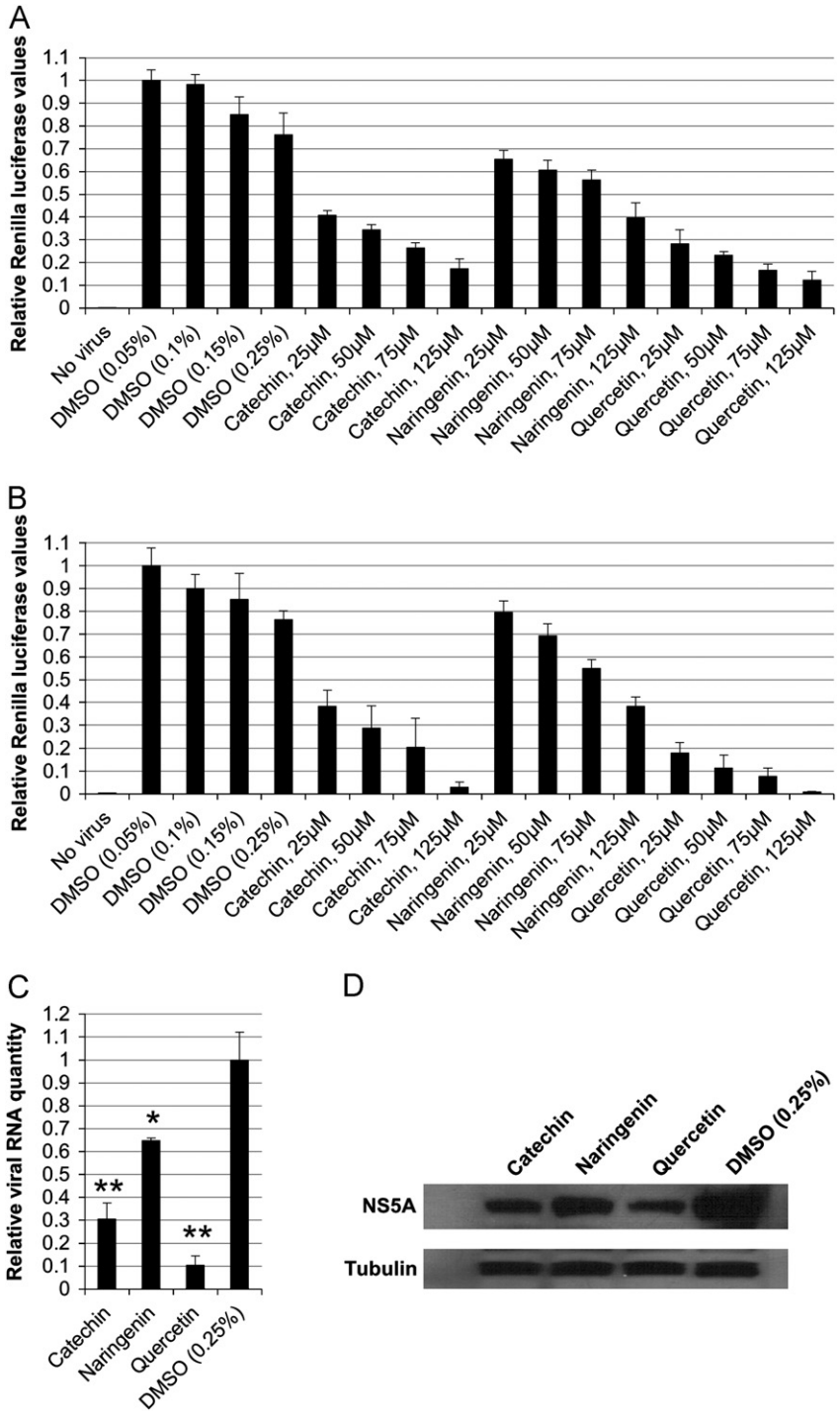

Fig. 4. Dose dependent antiviral activity of catechin, naringenin, and quercetin. (A) Huh-7.5 cells were infected with the *Renilla* reporter virus and immediately treated with a concentration range of 25–125 μM of each bioflavonoid for 72 h, followed by measuring luciferase activity. (B) Luciferase assays performed on cells infected with the concentrated supernatants of the cells in Fig. 4A. Huh-7.5 cells were infected with supernatants and harvested 72 h later. (C) Bioflavonoid effect on viral genome levels. Huh-7.5 cells were infected with the reporter virus and treated with 125 μM bioflavonoid for 72 h followed by quantitative reverse-transcriptase PCR. (D) Bioflavonoid effect on viral protein levels. Western analyses for NS5A and loading control tubulin were performed on the same samples from part (C). (* and ** indicate $P < 0.05$ and $P < 0.005$, respectively. Error bars reflect standard deviation.)

bioflavonoids for 5 h. This limited time of bioflavonoid treatment is necessary to minimize the effect of bioflavonoids on viral protein translation. Supernatants were removed and saved to determine infectious virion production (see below). After adding fresh medium, cells were subjected to three cycles of freeze/thaw to release intracellular virions. The medium was cleared of cellular debris and used to infect naïve cells. As shown in Fig. 7A, all three bioflavonoids significantly blocked infectious virion assembly; here catechin and naringenin demonstrated more potency compared to quercetin.

Luciferase assays of the 'control set' of cells showed no significant decrease in viral translation (Fig. 7B). BFA inhibition of secretion was verified by infection of naïve cells with the supernatants of the original infection. As shown in Fig. 7C, BFA treatment abolished viral secretion as expected, while significant viral secretion occurred when cells were treated with DMSO.

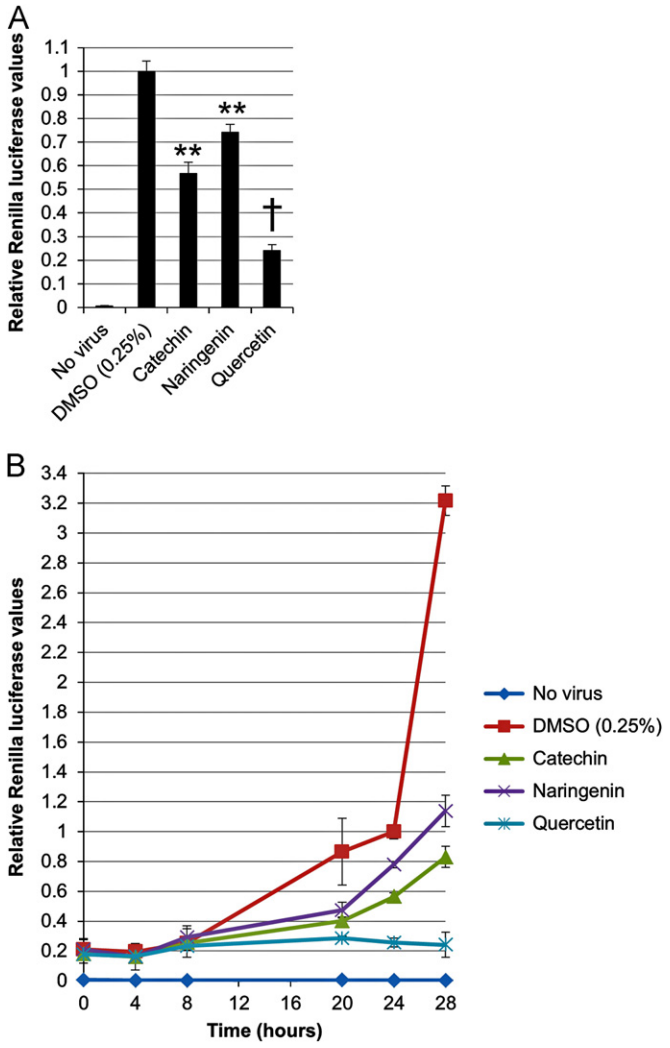

Fig. 5. Bioflavonoid effect on intracellular viral protein production. (A) Huh-7.5 cells were infected with the reporter virus and immediately treated with 125 μM bioflavonoid. Twenty hours later, luciferase levels were measured. (B) Luciferase assays performed in a similar way, but at different time points after infection. All values were normalized to the 24 h time point. (** and † indicate $P<0.005$ and $P<0.000005$, respectively. Error bars reflect standard deviation.)

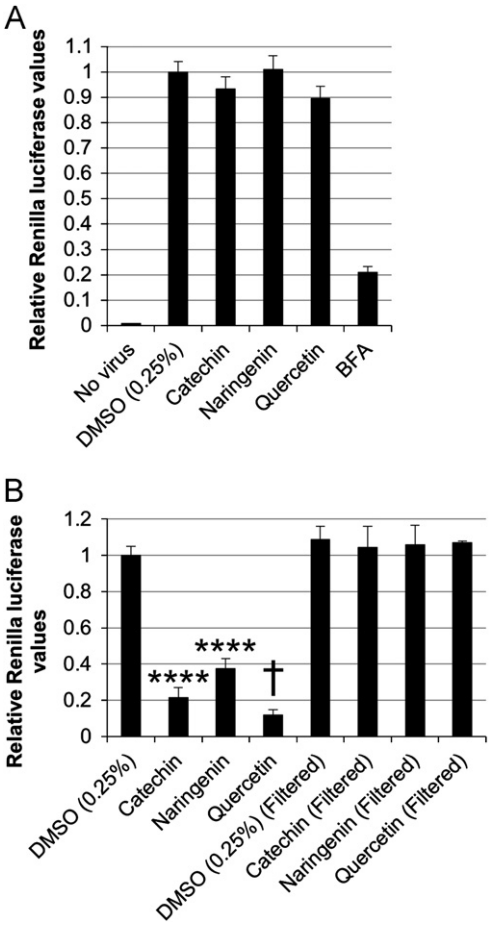

Fig. 6. Bioflavonoid effect on infectious virion secretion. (A) Huh-7.5 cells were infected and 24 h later, treated with 125 μM bioflavonoid for 5 h. Supernatants were immediately removed and concentrated 30-fold to remove approx. 97% of the bioflavonoids and used to infect naïve cells, followed by luciferase assays 72 h later. (B) Control experiment to determine if concentration effectively removed bioflavonoids from supernatants in part (A). Two sets of huh-7.5 cells were infected with the reporter virus. Subsequently, the supernatants of one set of cells were replaced with medium containing 125 μM bioflavonoid. For the other set of cells, the media containing 125 μM bioflavonoid were filtered and concentrated to remove the bioflavonoids. The cleared medium was then used to replace the culture supernatants. Seventy-two hours later, luciferase activity was measured. (**** and † indicate $P<0.00005$ and $P<0.000005$, respectively. Error bars reflect standard deviation.)

Quercetin blocks NS5A-augmented IRES-mediated translation, whereas naringenin and catechin exhibit mild activity

We tested catechin, naringenin, and quercetin in a previously described cell culture-based bicistronic reporter system to measure levels of viral internal ribosomal entry site (IRES)-mediated translation (Gonzalez et al., 2009). This reporter consists of a *Renilla* luciferase (RLuc) open reading frame (ORF) and a *Firefly* luciferase (FLuc) ORF driven by a 5′-cap and HCV IRES, respectively (Fig. 8A). The ratio of *Firefly* to *Renilla* luciferase values (FLuc/RLuc) reflects the levels of HCV IRES-mediated translation. We have previously shown that quercetin suppresses the NS5A-driven increase in IRES-mediated translation (Gonzalez et al., 2009). In this study, 293T cells were transfected with the reporter construct and either NS5A or GFP (control) and treated with 125 μM bioflavonoid for 72 h after which, cells were assayed for dual luciferase activity. All bioflavonoids significantly decreased IRES-mediated translation compared with DMSO (Fig. 8B). Quercetin completely blocked NS5A-augmented IRES activity in contrast to catechin and naringenin which demonstrated mild inhibition.

Quercetin strongly inhibits heat shock induced HSP70 expression compared to catechin, naringenin

Quercetin has been reported to inhibit HSP70 expression through different mechanisms (Elia and Santoro, 1994; Jakubowicz-Gil et al., 2005). Previously, we have shown HSP70 to form a complex with viral NS5A *in vivo* (Gonzalez et al., 2009). Further we recently showed that the NS5A/HSP70 complex is important for viral protein production *in vivo* and that disruption of this complex through a small peptide inhibitor results in a marked decrease in viral protein synthesis (Khachatoorian et al., 2012). Based on these observations, we hypothesized that the translation inhibitory effect of quercetin, as well as catechin and naringenin, may be mediated by inhibition of HSP70 expression. To test this hypothesis, huh-7.5 cells were treated with catechin, naringenin, quercetin, or DMSO (control) for 2 h and subjected to heat shock at 42 °C for 30 min and allowed to recover at 37 °C for 6 h. Western analysis of cellular lysates with antibody against HSP70 (specifically the HSPA1A isoform reported in our previous studies (Gonzalez et al., 2009; Khachatoorian et al., 2012))

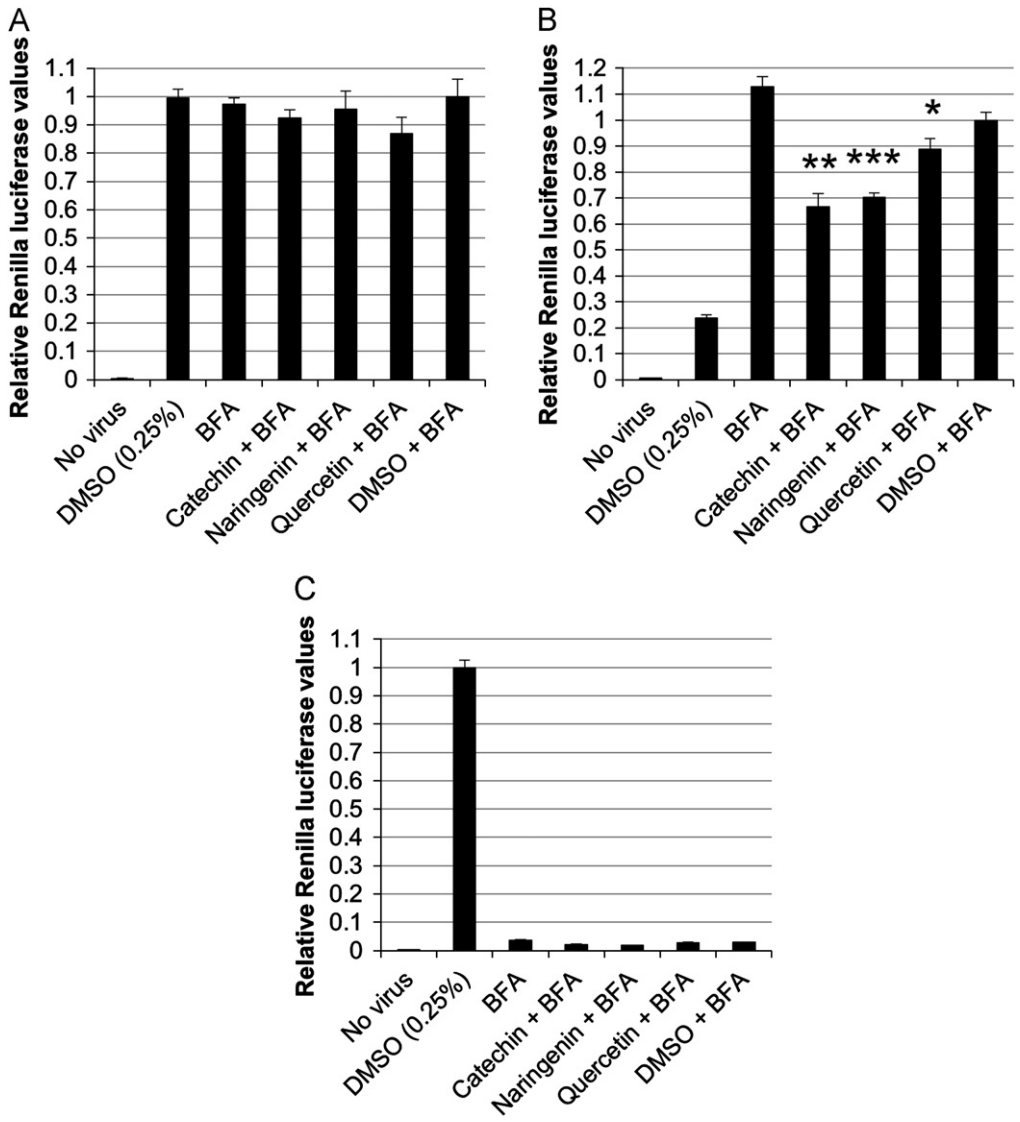

Fig. 7. Bioflavonoid effects on assembly. (A) Huh-7.5 cells were infected and immediately treated with 0.1 μg/ml of Brefeldin A (BFA). Thirty-one hours later, cells were treated with 125 μM bioflavonoid for 5 h after which, the medium was removed and saved for further analysis, and cells were washed twice with PBS to remove bioflavonoids. Fresh medium was added, and cells were subjected to three cycles of freeze/thaw. After clearing cellular debris, supernatants were used to infect naïve cells followed by luciferase assays 72 h later. All values were normalized to 'DMSO+BFA'. (B) Control experiment to determine if bioflavonoids effect viral protein translation in the conditions of part (A). Huh-7.5 cells were infected and treated with BFA and bioflavonoids exactly as described in part (A). Immediately after the 5-h bioflavonoid treatment, cells were washed and lysed, and *Renilla* luciferase assay was performed. (C) Control experiment to determine the effect of BFA on viral secretion in the original part (A) assembly assay. The supernatants saved from the original culture were concentrated 30-fold to remove bioflavonoids and used to infect naïve cells, followed by luciferase assays 72 h later. All values were normalized to DMSO treatment. (*, **, and *** indicate $P<0.05$, $P<0.005$, and $P<0.0005$, respectively. Error bars reflect standard deviation.)

demonstrated a marked decrease in HSP70 expression in quercetin treated cells (Fig. 8C and D). A slight decrease in HSP70 was seen for naringenin and catechin treatments (Fig. 8C and D) consistent with our IRES assay and viral protein production results above (Figs. 8B and 5, respectively).

Discussion

We have previously reported quercetin to efficiently block the NS5A-driven increase in IRES-mediated translation and HCV production (Gonzalez et al., 2009). In this study, we further analyzed the mechanisms of action of quercetin and a number of other bioflavonoids structurally related to quercetin.

Initially we tested seven bioflavonoids, including quercetin, for their cellular toxicity, and we found that genistein and luteolin were highly cytotoxic to huh-7.5 cells. Quercetin, naringenin, and silymarin displayed similar toxicity to the DMSO carrier, while catechin and epicatechin had the lowest toxicity and led to a significantly increased absorbance in MTT assays. We conclude that genistein and luteolin are not suitable as anti-HCV treatments due to their significant cytotoxicity at these concentrations.

Next, we screened the remaining five bioflavonoids for their antiviral activity. Catechin, naringenin, and quercetin significantly blocked virus production, while silymarin did not have any effect. Silymarin has previously been shown to inhibit HCV infection in tissue culture primarily through blocking viral entry (Wagoner et al., 2010). We did not see this effect. One possible reason is that we added the compounds after the 3-h infection time, which would significantly reduce the effect of an entry blocker. Further, the concentration we used was fairly low (25 μM). Others have

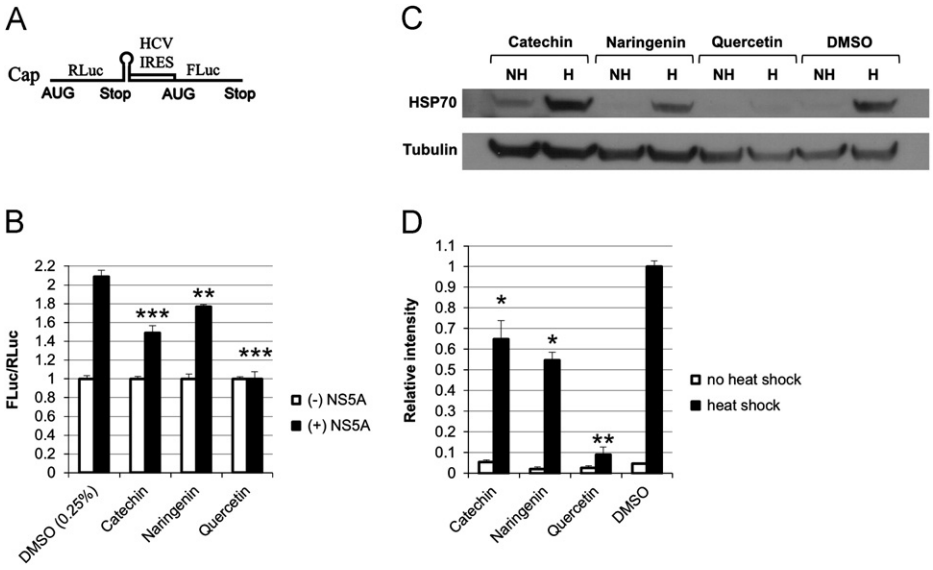

Fig. 8. Bioflavonoid effects on IRES-mediated translation. (A) Schematic of the bicistronic reporter construct used to measure IRES-mediated translation. *Renilla* luciferase (RLuc) and *Firefly* luciferase (FLuc) are driven by a 5′ cap and the HCV IRES, respectively. *Firefly* to *Renilla* ratios reflect changes in IRES-mediated translation. (B) Bioflavonoid effects on IRES-mediated translation. 293T cells were transfected with the IRES reporter construct and either NS5A or GFP. Twelve hours later, cells were treated with 125 μM bioflavonoid, followed by luciferase assays 72 h later. (C) Western analysis of the effect of bioflavonoids on HSP70 levels after heat shock. Huh-7.5 cells were treated with 125 μM bioflavonoids for 2 h and subjected to heat shock at 42 °C for 30 min. After 6 h of recovery at 37 °C, cells were lysed, and Western analysis was performed with antibody against HSP70 (HSPA1A). (D) Densitometry of the Western blot in panel (C). HSP70 quantities were normalized to tubulin. (*, **, and *** indicate $P<0.05$, $P<0.005$, and $P<0.0005$, respectively. Error bars reflect standard deviation.)

shown approximately 25% reduction in virus when treating J6/JFH infection (the same backbone as used in this study) when treating at 80 μM (Wagoner et al., 2010).

Epicatechin, a diastereoisomer of catechin, led to increased virus production compared with DMSO, and is, therefore, not suitable as an antiviral agent. Recently it was shown that a dimer of catechin and epicatechin can block HCV pseudotype proliferation (Li et al., 2010). Our finding that catechin and epicatechin have opposite effects on cellular viral levels implies that catechin may be the element in this dimer that displayed antiviral effects. We are currently investigating the opposite effects of catechin and epicatechin on virus production as well as their shared increased absorbance seen in MTT assays. As shown in Fig. 1, the di-hydro-benzopyran backbone of catechin and epicatechin possesses a di-hydroxyphenyl and a hydroxyl group on the pyran moiety. These two groups are oriented differently in three dimensional space; in catechin they are located on the same side of the backbone, while in epicatechin they point in opposite directions. We speculate that the orientation of these side chains may be responsible for the opposite effects of catechin and epicatechin on viral protein translation and are currently investigating this possibility. We have shown previously that viral protein translation is mediated in part by a complex of NS5A and HSP70 (Gonzalez et al., 2009; Khachatoorian et al., 2012). Considering our finding that catechin can effect HSP70 expression, it may be possible that the orientation of these side chains determines the effect of catechin and epicatechin on HSP70 expression potentially through their differential interactions with the HSP70 transcription factor.

We chose catechin, naringenin, and quercetin for further analysis because of their antiviral activity. All three bioflavonoids significantly block cellular viral levels in the HCVcc system. Quercetin displays a far more potent effect than catechin and naringenin. Catechin inhibits viral translation more than naringenin, and we speculate that this results in the better long-term viral attenuation by catechin compared with naringenin (Fig. 4). We also used the HCV IRES bicistronic reporter assay system to show that the translation inhibitory effect of these flavonoids is NS5A dependent as there was no change in IRES-mediated translation when GFP was used (instead of NS5A) as a control. This result is consistent with our previous findings on the mechanism of NS5A/HSP70 complex-driven IRES-mediated translation of viral proteins (Gonzalez et al., 2009; Khachatoorian et al., 2012).

Intracellular infectious virion assembly is also significantly blocked by catechin and naringenin and to a lesser extent by quercetin. Naringenin has also been previously reported to block virion assembly using a different assay (Goldwasser et al., 2011). Infectious virion secretion is not significantly affected by catechin, naringenin (in agreement with a previous report (Goldwasser et al., 2011)) and quercetin.

Furthermore, we have shown that catechin, naringenin, and quercetin effect induction of HSP70 in cells that are subjected to heat shock. In particular, quercetin has a far stronger inhibitory effect on HSP70 expression. These results support our hypothesis that bioflavonoids mediate their antiviral effects at least in part by blocking heat shock protein (HSP) expression and underscore the role of HSPs in HCV life cycle.

Current HCV treatments are limited and display significant side effect and suboptimal sustained virological response (SVR). For these reasons, it is necessary to identify/develop additional antiviral therapies that could be used in place of pegylated interferon-α (PEG-IFN) and ribavirin or as adjunct therapies to increase the SVR. In this study, we have demonstrated the significant antiviral activity of catechin, quercetin and naringenin. Therefore, these bioflavonoids may be candidates for HCV therapy and may be beneficial for patients unable to receive PEG-IFN therapy. Furthermore, because of the different mechanisms of action of these bioflavonoids, combining them may allow for synergistic antiviral activity resulting in better suppression of HCV.

Materials and methods

Bioflavonoids

Quercetin (Sigma-Aldrich, 00200595-50MG), catechin (Sigma-Aldrich, C1251-5G), naringenin (Sigma-Aldrich, N5893-1G), epicatechin (Sigma-Aldrich, E4018-5MG), silymarin (Sigma-Aldrich,

S0292-10G), genistein (Sigma-Aldrich, G6649-5MG), and luteolin (Sigma-Aldrich, L9283-10MG).

Cell culture

Cell lines Huh-7.5 and 293T were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (Mediatech, 10-013-CM) supplemented with 10% fetal bovine serum (Omega Scientific, FB-01) and 2 mM L-glutamine (Invitrogen, 25030). 293T cells were purchased from ATCC (CRL-11268). Huh-7.5 cells were a kind gift from Charles Rice (The Rockefeller University, New York, NY) (Blight et al., 2002).

Cell viability

Cell viability was determined using MTT Cell Proliferation assay (ATCC, 30-1010K).

Plasmid constructs

The HCV IRES reporter plasmid and the NS5A and GFP retroviral expression vectors pMSCV-NS5A-FLAG and pMSCV-GFP, respectively, have been previously described (Gonzalez et al., 2009). An intra-genotype 2 chimeric monocistronic reporter virus, pNRLFC based on pJ6/JFH-C parental virus has been described previously (Arumugaswami et al., 2008). For the current study, we have used a chemically synthesized plasmid pFNX-RLuc (having similar sequences to pNRLFC) for construction of recombinant virus. The pRL-TK (Promega, E2241) plasmid expresses *Renilla* luciferase.

Infectious virus production

pFNX-RLuc was *in vitro* transcribed, and the purified RNA was electroporated into huh-7.5 cells to generate infectious viral supernatant as previously described (Arumugaswami et al., 2008).

Viral assays

All viral assays were done using the HCV reporter virus and with the same titer and multiplicity of infection as described previously (Gonzalez et al., 2009; Khachatoorian et al., 2012). **Intracellular viral protein production:** Huh-7.5 cells were infected for 3 h, and cells were harvested at the indicated time points. Luciferase activity was measured using the *Renilla* Luciferase Assay System (Promega, E2820). **Infectious virion secretion:** The supernatants from the above cultures were concentrated 30-fold by using Amicon Ultra-0.5 mL Centrifugal Filters (Millipore, UFC510096) to remove excess bioflavonoids and used to infect naïve cells for 3 h. Cells were harvested 72 h later, and *Renilla* luciferase activity was measured. **Intracellular infectious virion assembly:** Huh-7.5 cells were infected for 3 h, and supernatants were removed at indicated time points. Cells were washed with PBS, and fresh medium was added. The cultures were subjected to three cycles of freeze–thaw to release assembled viral particles. These suspensions were cleared of cellular debris and used to infect naïve cells for 3 h. Subsequently, cells were harvested 72 h post infection, and luciferase activity was assayed.

Quantitative reverse-transcriptase PCR

Huh-7.5 cells were infected with the *Renilla* reporter virus for 3 h. Seventy-two hours post infection, cells were harvested, and total RNA was extracted using RNeasy Mini Kit (Qiagen, 74104). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, 1708891). Quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System with 2x SYBR Green Master Mix (Diagenode, GMO-SG2x-A300) in 25 μL reactions. The real-time PCR cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s each as well as a final dissociation stage of 95 °C for 15 s and 60 °C for 1 min. The primers for the viral genome were derived from the 5′-non-coding region and were CTGGGTCCTTTCTTGGATAA and CCTATCAGGCAGTACCACA. HCV RNA levels were normalized to the housekeeping gene actin using the primers CCAACCGCGAGAAGATGA and CCAGAGGCGTACAG GGATAG.

IRES reporter assay

293T cells were treated with 125 μM bioflavonoids. Two hours later, cells were transfected with the HCV IRES reporter plasmid and either pMSCV-NS5A-FLAG or pMSCV-GFP. All transfections were done using Fugene6 (Roche, 11814443001). Forty-eight hours post transfection, *Renilla* and *Firefly* luciferase activity were determined using Dual Luciferase Assay System (Promega, E1910).

Antibodies

NS5A (Abcam, ab20342), HSP70 (Santa Cruz Biotech, C92F3A-5), and tubulin (abcam, ab6160).

Densitometry

Western blot images were analyzed by ImageJ v1.45s software according to software instructions.

Statistical analysis

Error bars reflect the standard deviation. *P* values were determined by student t-test.

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