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Functional evaluation of synthetic flavonoids and chalcones for potential antiviral and anticancer properties

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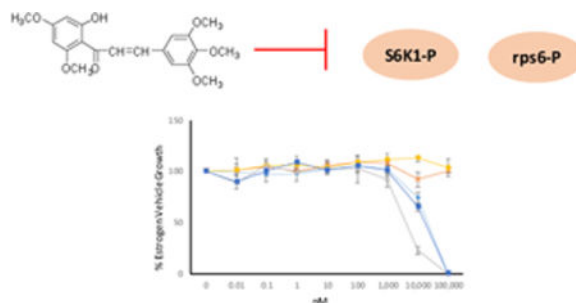
Abstract

Flavonoids, stilbenes, and chalcones are plant secondary metabolites that often possess diverse biological activities including anti-inflammatory, anti-cancer, and anti-viral activities. The wide range of bioactivities poses a challenge to identify their targets. Here, we studied a set of synthetically generated flavonoids and chalcones to evaluate for their biological activity, and compared similarly substituted flavonoids and chalcones. Substituted chalcones, but not flavonoids, showed inhibition of viral translation without significantly affecting viral replication in cells infected with hepatitis C virus (HCV). We suggest that the chalcones used in this study inhibit mammalian target of rapamycin (mTOR) pathway by ablating phosphorylation of ribosomal protein 6 (rps6), and also the kinase necessary for phosphorylating rps6 in Huh7.5 cells (pS6K1). In addition, selected chalcones showed inhibition of growth in Ishikawa, MCF7, and MDA-MB-231 cells resulting an IC₅₀ of 1–6 µg/mL. When similarly substituted flavonoids were used against the same set of cancer cells, we did not observe any inhibitory effect. Together, we report that chalcones show potential for anti-viral and anti-cancer activities compared to similarly substituted flavonoids.

Graphical abstract

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Keywords

Chalcone; HCV; Cancer; mTOR; rps6

Bioactive molecules, chalcones and flavonoids, present in plants are key components in therapeutic studies against infectious diseases, cancer, and metabolic disorders. The large plethora of available flavonoids and chalcones, studied so far indicate a wide spectrum of bioactivity, and sometimes promiscuous targets that resulted due to their effect on overlapping pathways. Many of these natural compounds possess anti-viral activity against several viruses including the hepatitis C virus (HCV). Flavonoids such as naringenin, quercetin, and leuteolin, and stilbenes such as tamoxifen – a chemotherapeutic drug for breast cancer – have been evaluated for its anti-HCV effect.¹ In addition, several naturally occurring and synthetic flavonoids have been suggested to have anti-cancer activity in various cell types.² However, the diverse bioactivity of these compounds pose a challenge to identify their individual targets, and study their structure function relationships. Prolonged HCV infection in the liver results in cirrhosis, hepatocellular carcinoma, and often leads to liver transplantation and other medical complications. In the absence of a vaccine, drugs are designed to target either viral proteins, or host factors necessary to support the viral life cycle inside the host cell.^{3–5} The current treatment involve oral intake of sofosbuvir.^{6, 7} But high expense for sofosbuvir treatment inspires a thorough look at available potential drug-like molecules that are easily extracted from plants, or their chemically synthesized derivatives (Table 1).^{7–9} HCV is a positive strand RNA virus.¹⁰ HCV encoded viral nonstructural protein 5A (NS5A) has been implicated in many steps of viral life cycle including RNA replication, RNA translation, and viral assembly.¹¹

During viral translation NS5A activates mammalian target of Rapamycin (mTOR) pathway by disrupting the interaction between FK506-binding protein 38 (FKB38) and mTOR.^{12, 13} Activated mTOR initiates phosphorylation of ribosomal protein S6 kinase beta-1 (S6K1) protein, which eventually phosphorylates ribosomal protein 6 (rps6).¹⁴ Expression of NS5A increases the level of phosphorylated pS6K1. Additionally, cells infected with genotype 2a HCV virus show significant increase in p-rps6 at Ser235/236.¹² Both HCV infection and NS5A expression enhance the assembly of cap-dependent translational complex on the mRNA through mTOR activation.¹² This enhanced translational complex increases protein expression from specific set of mRNAs, especially expression of ribosomal proteins, without changing the level of global translation.^{12, 13}

Flavonoids and other plant metabolites, inhibited HCV propagation at different stages including virus entry, and replication.¹⁵ To our knowledge the study of chalcones as the HCV inhibitor is limited. On the contrary chalcones have long been studied for its potential in various diseases including cancer.^{16–19} Recent literature described mTOR as a potential target for hepatocellular carcinoma.²⁰ Increased mTOR activity is altered in prostate cancer,^{19, 21} in breast and ovarian cancer,²² tumorigenesis,²³ and in hepatocellular carcinoma.²⁴ Several anticancer drugs have been identified as mTOR inhibitors,^{20, 25–28} including the breast cancer.^{26, 29} Since other mTOR inhibitors have been implicated in anti-cancer therapy, we compared substituted chalcones and flavonoids against hepatitis C virus, and in breast and ovarian cancer cells.

We screened eighteen synthetic flavonoids and chalcones (Table 1) against HCV infected cells to investigate any anti-HCV function. We infected Huh7.5 cells with genotype 2a strain (JHF1-FLAG), and co-treated with substituted flavonoids and chalcones at a concentration up to 50 μM .^{30–32} We analyzed both a viral protein (NS3) and a cellular protein (GAPDH), and evaluated the ratio of NS3 to GAPDH during the 72 hour incubation period. Although flavonoids naringenin, quercetin, and leuteolin are identified to interfere with the HCV life cycle at different stages, none of the flavonoids used in this study showed anti-HCV effect below 50 μM concentration in our assay.⁸ In contrast, most of the substituted chalcones (Table 1: NM1, NM2, NM5, NM6, NM7, NM8) exhibited varied degree of inhibition of HCV as measured by a reduction in the ratio of NS3 to GAPDH relative to cells treated with DMSO (Supplementary Figure S1). Our initial experiments verified that functionally active compounds do not inhibit an earlier step of viral life cycle including entry when viral infection was initiated with genomic RNA in the presence of active chalcones (Supplementary Figure S2). Since flavonoids and chalcones used in this study do not affect an early step in HCV life cycle, we investigated if these compounds are active in cells containing subgenomic replicon that are only able to replicate a partial viral genome followed by translation of several viral proteins required for replication.^{33, 34}

We treated both genotype 2a (SGR2a) and 1b (GS5) containing cells with selected chalcones for 72 hours and analyzed the ratio of NS3 to GAPDH (Figs. 1a and 1b). Treatment of GS5 cells had a significantly greater inhibition than SGR2a. Furthermore, we also noticed that in both cell types bulky substituents on the benzene ring ($-\text{H}$, $-\text{CH}_3$ vs $-\text{C}_5\text{H}_{11}$ in NM1, NM2 and NM3 respectively) caused significantly lower anti-viral activity in both genotypes (Figs 1a and 1b, right panel and S1) suggesting a structure–function relation between active chalcone and its target. NM1 and NM5 consistently showed significant inhibition of viral production, hence were selected for the rest of the study to understand the target of chalcone mediated inhibition of HCV. In order to understand concentration dependence of chalcone derivatives, we treated replicon cells with increasing concentrations of NM1, and NM5 for 72 hours (Figs. 2a and b). Since chalcones show inhibition of HCV in replicon cells, we extracted total RNA from cells and quantified viral RNA level by quantitative PCR using HCV specific primers (Supplementary Materials). To our surprise, HCV RNA level, although decreased modestly, remained unchanged over a concentration range that spans from 0.1 μM to 10 μM . Since the RNA level remained mostly unchanged, we analyzed the viral protein level by comparing NS3 to GAPDH ratio from both genotypes 2a and 1b cells. Unlike the RNA level, both genotypes showed 50% inhibition of NS3 protein at 5 μM

concentration (Figs. 2c, d, and e). Surprisingly, at a concentration below 5 μM (at 0.5 and 1 μM), both NM1 and NM5 showed a consistent and significant increase in viral protein (150%) above the control treatment (DMSO, 100%) before reducing to 50% at 5 μM concentration (Figures 2c, d, and e). NM6 treatment also showed similar outcome (Supplementary figure S3). Therefore, viral translation, but not RNA replication is targeted by chalcones. To examine if all HCV inhibitors function by first stimulating HCV protein production before a significant inhibition, we used cyclosporine A (CsA), a well-studied inhibitor of HCV. SGR2a cells were treated with increasing concentrations of CsA until NS3 level was significantly reduced.^{35, 36} When the ratio of viral protein (NS3) was compared with β -Actin at different concentrations of CsA, a gradual decrease in the NS3 level was observed without ever reaching a value higher than DMSO treated cells (Supplementary figure S4), suggesting that the chalcones use a different mode of inhibition than CsA.

A thorough look at the current literature suggests that HCV infected cells regulate the level of mTOR pathway to increase phosphorylation of ribosomal proteins.¹³ A previous study suggested that cells infected with 2a HCV virus in normal growth media (10% FBS) showed significant increase in phosphorylation of rps6 at Ser235/236 with modest increase in the basal level of rps6. Since chalcones effectively blocked viral translation, we asked if chalcones may block phosphorylation of rps6 protein which happens through the mTOR pathway. We analyzed the level of total and phosphorylated rps6 in SGR2a replicon cells treated with increasing concentration of NM5. We observed a modest decrease in the total rps6 but a significant drop in the level of the phosphorylated rps6 (Fig. 3a). A ratio of two showed a substantial decrease in the phosphorylated rps6 to below 50% relative to either GAPDH or total rps6 at the chalcone concentration of 0.5–1 μM (Fig. 3a, right panel). Similar effect was observed upon NM1 treatment (data not shown). Surprisingly, we observed an increase in the phosphorylated rps6 under a low concentration of chalcone (0.5 and 1 μM), this result is parallel to increase in viral protein concentration under the same chalcone concentration suggesting phosphorylated rps6 level may regulated viral NS3 level. mTOR function is defined by two separate signaling pathways. mTORC1 promotes protein synthesis through enhanced phosphorylation of S6K1 protein that serves as a kinase and is ultimately responsible for phosphorylating rps6. It is suggested that the mTORC2, a less studied pathway, influences cell growth and proliferation through phosphorylating AKT1 protein.³⁷ To validate that the mTOR pathway is actually affected, we analyzed phosphorylated S6K1 protein in the treated cells.

Results showed a sharp decrease in p-S6K1 at 0.1 μM concentration of chalcones (Fig. 3a). We examined if the effect of chalcones on S6K1 and rps6 is related to the presence of HCV (or NS5A), and incubated naïve Huh7.5 cells with increasing concentration of NM5. Results conclude that the effect of NM5 (Fig. 3b) on mTOR pathway is independent of the presence of HCV proteins. We tested if this is a unique property of NM5 and other chalcones, and analyzed the cells treated with CsA. Cell carrying subgenomic replicon type 2a (SGCR2a) was treated with an increasing concentration of CsA until NS3 level is diminished to 10% relative to the DMSO treated cells (Supplementary figure S4). p-rps6 remained constant throughout the concentration range (Fig. 3c). Chalcone mediated inhibition of HCV translation in cells with diminished level of phosphorylated rps6 suggest that HCV translation is severely affected by low level of phosphorylated rps6, while the cellular

Overall, our results have two significant outcomes. One, inhibition of phosphorylated rsp6 and S6K1 by chalcones dampen HCV translation. These results can be used to design and analyze drugs that are more effective. Second, compared to respective flavonoids, chalcones are more effective anti-HCV agents and anti-cancer compounds in Ishikawa, MCF7, and MDA-MB-231 cells. Chalcones block phosphorylation of rsp6 and S6K1 potentially through inhibition of mTOR (Fig. 3), and are effective anti-cancer agents that are comparable to Tamoxifen and its derivatives (Fig. 4). Although effective concentration of chalcones studied in this work are relatively high against HCV (5 μ M), effective concentration necessary to inhibit growth in cancer cells are comparable to established cancer drugs. Chalcones and flavonoids differ by the closed ring structure present in the flavonoid but not in chalcones providing more flexibility in the latter during target binding. Interestingly, in our study all chalcones showed varied level of activity while flavonoids did not show any inhibition of HCV translation at the concentrations used in this study. Similarly, chalcones, not flavonoids, effectively inhibited growth in cancer cells used in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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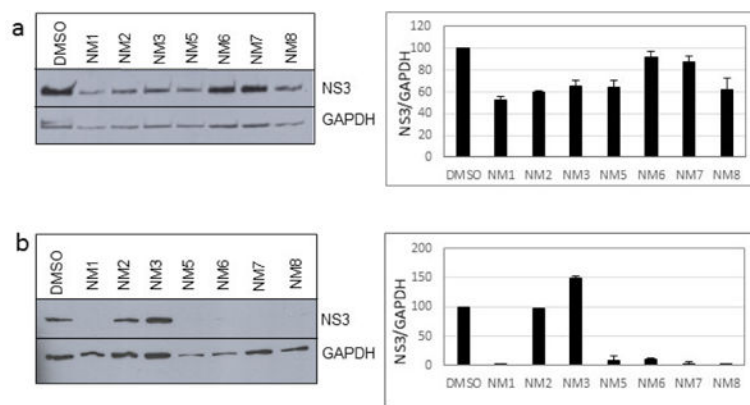


Figure 1. Inhibition of HCV replicon cell by chalcones. (a) SGR2a and (b) GS5 cells were treated for 72 hours with chalcones at 10 μ M concentration. Cells were collected and extracted proteins were analyzed using Western Blot. Right side panels show quantitative analysis of the western blot on the left and repeats using Image J.

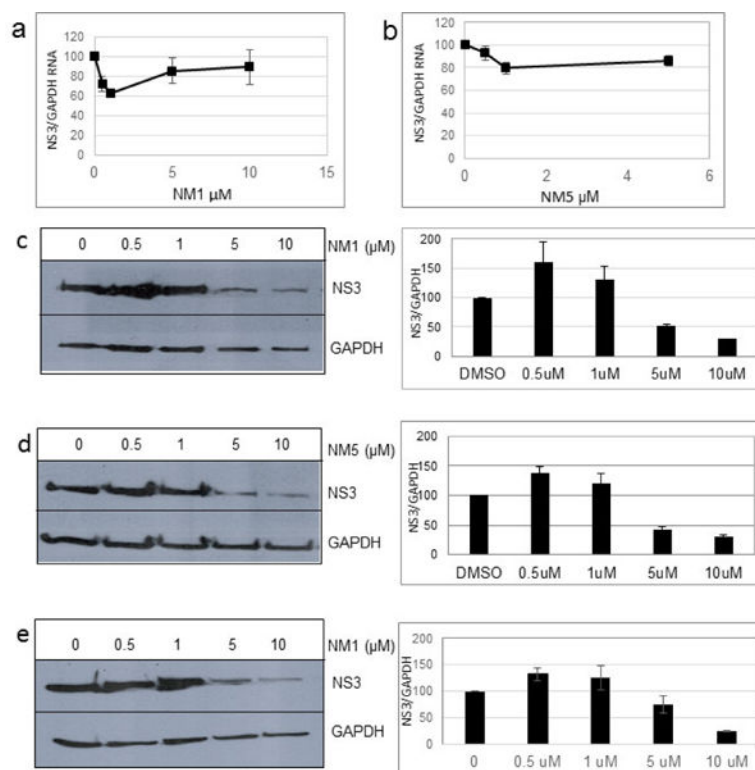


Figure 2. Concentration dependent inhibition of HCV. SGR2a cells were treated for 72 hours with (a) NM1 and (b) NM5 and GS5 cells were treated with (c) NM1, followed by isolation of RNA. HCV RNA and GAPDH mRNA were quantified using qPCR. Proteins from (a), (b) and (c) were analyzed by Western Blot. Right side panels for (c), (d) and (e) show quantitative analysis of the western blot on the left and their respective repeats using Image J.

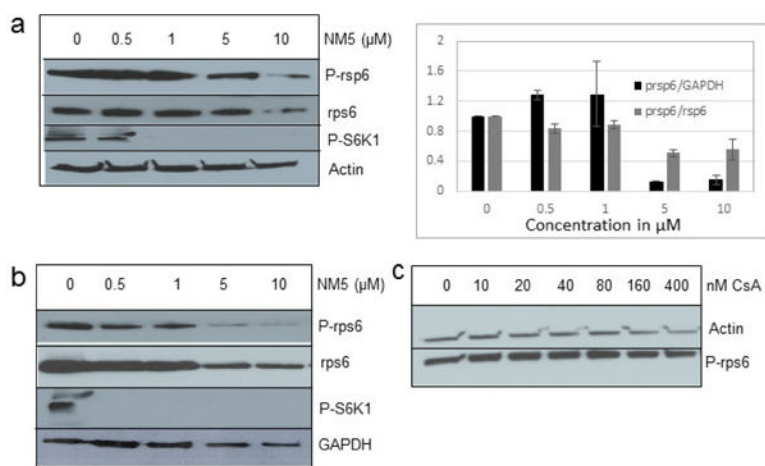


Figure 3.

Chalcones repress phosphorylation of rps6. (a) Left: SGR2a cells were incubated with NM5 for 72 hours followed by analysis of rps6, p-rps6, and GAPDH. Right: Quantitative analysis of western blot of left panel and repeats using ImageJ. (b) Huh7.5 cells were incubated with NM5 followed by analysis of p-rps6, rps6, and actin. (c) SGR2a cells were incubated with increasing amount of CsA for 72 hours, followed by analysis of p-rps6 and actin.

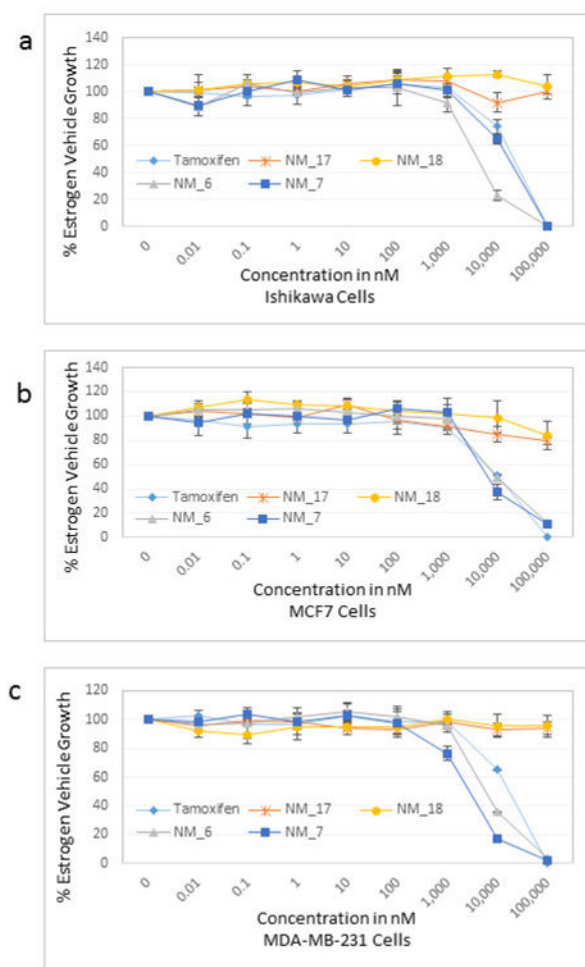


Figure 4. Analysis of estrogen mediated growth inhibition by chalcones and flavonoids in cancer cells. (a) Ishikawa, (b) MCF-7, and (c) MDA-MB-231 cells were plated in 96 well plates and were incubated with 10 nM estrogen and indicated amount of tamoxifen, NM6, NM7, NM17, and NM18 for 72 hrs. Cell were incubated with Cell-Glow-Titer for 12 minutes at room temperature before immunofluorescence measurement.

Table 1

List of chalcones and flavonoids.

NM_1		NM_10	
NM_2		NM_11	
NM_3		NM_12	
NM_4		NM_13	
NM_5		NM_14	
NM_6		NM_15	
NM_7		NM_16	
NM_8		NM_17	
NM_9		NM_18	

Table 2IC₅₀ value comparing chalcones, flavonoids, and tamoxifen in cancer cells.

Compound	IC ₅₀ (µg/mL)		
	Ishikawa	MCF-7	MDA-MB-231
NM_6	5.85	3.30	1.98
NM_7	1.43	2.24	0.99
NM_17	>36.72	>36.72	>36.72
NM_18	>35.73	>35.73	>35.73
Tamoxifen	7.87	3.99	7.85
Raloxifene-Hydrochloride	10.53	0.98	11.21
4-Hydroxy Tamoxifen	3.57	0.95	6.51

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