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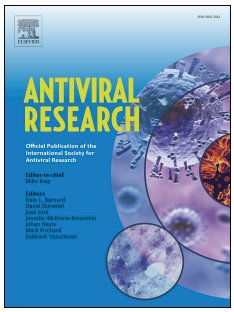

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Caffeic acid inhibits HCV replication via induction of IFNα antiviral response through p62-mediated Keap1/Nrf2 signaling pathway

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Abstract

Hepatitis C virus (HCV) infection and its related liver disease have constituted a heavy burden worldwide. It had been reported that Drinking coffee could decrease mortality risk of HCV infected patients. Caffeic Acid (CA), the Coffee-related organic acid could inhibit HCV replication, however, the detailed mechanism of CA against HCV is unclear. In this study, we showed that CA could notably inhibit HCV replication. Mechanism study demonstrated that CA could induce HO-1 expression, which would trigger the IFNα antiviral response, and the antiviral effect of CA was attenuated when HO-1 activity was inhibited by SnPP (an HO-1 inhibitor). CA could also increase erythroid 2-related factor 2 (Nrf2) expression. When Nrf2 was knocked down by specific siRNA, HO-1 expression was concomitantly decreased while HCV expression was restored. Further study indicated that kelch-like ECH-associated protein 1 (Keap1) expression was decreased by CA in a p62/Sequestosome1 (p62)-dependent way, which would lead to the stabilization and accumulation of Nrf2. The decrease of

Keap1 was restored when p62 was silenced by specific p62 siRNA, suggesting p62 was required for CA-mediated Keap1 downregulation. Taken together, the results demonstrated that CA could modulate Keap1/Nrf2 interaction via increasing p62 expression, which will lead to stabilization of Nrf2 and HO-1 induction and elicit IFNα antiviral response to suppress HCV replication.

Key Words: Caffeic acid; Keap1; Nrf2; HO-1; p62.

Highlights:

- CA inhibits HCV replication by inducing HO-1 expression and thus eliciting IFNα antivral response
- CA activates Nrf2 to induce HO-1 expression
- CA increases p62 expression and thus downregulates Keap1 expression to activate Nrf2
- Lay the foundation for a wider application of CA and the potential optimization of CA structure

Abbreviations

CA, caffeic acid; HCV, hepatitis c virus; IFNα, interferon α; p62, Sequestosome1/p62; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; Keap1, kelch-like ECH-associated protein 1; HO-1, heme oxygenase-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OAS, 2,5-oligoadenylate synthetase; PKR, protein kinase R.

1. Introduction

HCV has posed a heavy burden all over the world, with approximately 170 million people being chronically infected. HCV belongs to the *Flaviviridae* family and is a single positive-strand RNA virus of 9.6 kb in length, which contains a long open reading frame (ORF) flanking by 5' and 3' untranslated regions (UTRs). The genome can be translated via an internal ribosome entry site (IRES) into a polypeptide, which will be later processed by host and viral enzymes to ten proteins, including core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Hoffman and Liu, 2011). The landscape of HCV treatment has been dramatically revolutionized in recent years (Kardashian and Pockros, 2017). A newly approved drug, Harvoni has shown an

optimistic antiviral activity against different HCV genotypes (Mullins et al., 2015). Despite all these achievements, several minor side effects still persist: fatigue, yellow fever, especially the high cost, which blocked most patients' access to these effective regimens (Braillon, 2015). Besides, these regimens have merely been applied for a short term and it needs more time to confirm the long-term effects. Therefore, more options that are more pan-genotypic, more tolerated, cheaper and with a shorter treatment duration are needed to enrich the pipelines of HCV treatments.

CA is an organic compound found in almost all plants (Boerjan et al., 2003). CA is believed to exert a variety of pharmacological effects in the body, including antioxidant (Gulcin, 2006), antimicrobial (Utsunomiya et al., 2014), antiviral (Wang et al., 2009) and anti-inflammatory effects (Chao et al., 2010). A recent study has shown that coffee drinking could decrease the mortality risk in HCV-HIV coinfected patients (Carrieri et al.), which could be possibly attributed to the role of CA in coffee. Numerous studies has proved a correlation between oxidative stress and HCV infection, and antioxidants have been regarded as a choice for HCV treatments (Choi, 2012). HO-1 is an essential enzyme involved in antioxidant pathway (Otterbein et al., 2003) and is responsible for cleaving heme to form biliverdin, carbon monoxide, ferrous iron. Biliverdin is proved to possess anti-HCV effects through eliciting IFNα antiviral response and impairing HCV NS3/4A proteinase activity (Lehmann et al., 2010; Zhu et al., 2010).

p62 is an ubiquitin binding protein involved in cell signaling, oxidative stress and autophagy (Bjørkøy et al., 2006; Komatsu et al., 2010; Seibenhener et al., 2007). P62 is found to interact with ubiquitin, providing a scaffold for several signaling proteins and triggering degradation of proteins through the proteasome or lysosome (Vadlamudi et al., 1996). Protein aggregates formed by p62 can be degraded by the autophagosome (Bjørkøy et al., 2005; Bjørkøy et al., 2006). Light Chain 3 (LC3) is identified as a subunit of microtubule-associated proteins and considered as indicators of autophagy (Kabeya et al., 2004) when the conversion of LC3-I to LC3-II, the lower migrating form occurs. P62 binds to autophagosomal membrane protein LC3, bringing p62-containing protein aggregates to the autophagosome (Pankiv et al.,

2007). P62 can also interact with Keap1, which is a cytoplasmic inhibitor of Nrf2, a key transcription factor involved in cellular responses to oxidative stress (Komatsu et al., 2010). Thus, accumulation of p62 can lead to an increase in Nrf2 activity.

Nrf2 is a transcription factor involved in the regulation of HO-1 regulation (Li et al., 2004). Under physiological conditions, Nrf2 is sequestered in the cytosol by Keap1, an adaptor of Cullin3-based E3 ligase (Cullin3) complex, and is ubiquitinated by Cullin3. Then the ubiquinated-Nrf2 is degraded by proteasome while the level of Nrf2 is maintained at a basal level (Nguyen et al., 2005; Pintard et al., 2004). However, under stressful or electrophilic conditions, Keap1 detaches from the complex and Nrf2 is released. Nrf2 accumulates in the cytoplasm and translocates into the nucleus, which will activate the related downstream gene expression. The free Keap1 will then degraded by autophagy mediated by the p62 and LC3 (Ichimura et al., 2013).

Previous studies have shown that CA could inhibit HCV replication through blocking HCV entry (Tanida et al., 2015), but detailed mechanism is not yet clarified. Here, we show that CA could inhibit HCV replication by increasing p62 expression through activating Keap1/Nrf2 pathway and triggering IFNα antiviral response.

2. Materials and methods

2.1. Cell culture and reagents

Huh7.5.1 was maintained in the DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Con1（HCV subgenomic replicon system, genotype 1b）(Lohmann et al., 1999)cells were cultured in DMEM supplemented with 0.5mg/ml geneticin. All cells were incubated at 37°C with 5% of CO_2.

Antibodies, anti-NS5B, anti-β-actin antibodies were purchased from Abcam (United Kingdom); anti-HO-1, anti-Nrf2, anti-Keap1, anti-p62, anti-phosphorylated-p62, anti-LC3 and anti-Cullin E3 ligase(Cullin3) antibody were purchased from Cell Signaling Technology (USA). Caffeic acid was purchased from Sigma Aldrich (USA); tin protoporphyrin IX (SnPP), rapamycin, MG132 were purchased from Selleck (USA).

2.2. The amplification, purification, storage and infection of HCV virus

J399EM, a JFH-1 based adaptive strain in which an EGFP gene was inserted into NS5A (Han et al., 2009) is employed to infect Huh7.5.1 cells at a MOI of 0.01. Six hours later, viral supernatants were replaced with fresh medium. Cells were passaged every 3-5 days. Medium was harvested 8-9 days postinfection. Cell debris was removed by centrifugation and filtration.

Confluent cultures of cells were inoculated with J399EM medium at a MOI of 0.1. Six hours later, supernatants were replaced with fresh culture medium with or without compounds, and cultured for 3 days. Then the medium was removed and the absorbance was read at a microplate reader (PerkinElmer).

2.3. HCV subgenomic replicon assay

Confluent cultures of Con1 cells were treated with different doses of compounds for 72 hours. Then the intracellular RNA and proteins were extracted and subjected to reverse transcription and western blot respectively to evaluate the effect of CA on HCV replication.

HCV titration, infection

Endpoint dilution assays (EDA) were used to determine the virus titers (Zhong et al., 2005). For the EDAs, Huh7.5.1 cells were seeded into 96-well plates at a density of 1×10^4 cells/well. Viral supernatants were 10-fold diluted serially in complete growth medium and were used to infect the cells at an MOI of 0.1. Six hours later, supernatants were replaced with fresh medium. Following 3 days of incubation, the cells were monitored and EGFP fluorophores within cells were counted using a fluorescence microscope (Nikon).

2.4. Total RNA extraction, reverse transcription and quantitative PCR

Intracellular and extracellular RNA were extracted using QIAamp RNeasy kit and QIAamp Viral RNA Mini kit (Qiagen), respectively according to the manufacturer's protocols. RNA was subjected to reversed transcription and quantitative real time polymerase reaction (qPCR) using the ABI 7900HT Real-Time PCR System (UK). GAPDH gene was employed as the endogenous control for normalization in all qRT-PCR analyses. The primer sequences were listed below.

Target gene	Sequences
2a f	GCGCGCTCTAGACCCGCCCCTAATAGGGGCGACACTCCGCC
2a r	GGA AACCAAGCTGCCCATCA
1b f	GGA AACCAAGCTGCCCATCA
1b r	CCTCCACGGATAGAAGTTTA
GAPDH f	GGTATC GTG GAA GGACTCATGAC
GAPDH r	ATGCCAGTG AGCTTCCCGTTCAGC
IFN2 f	ATTCGTATGCCAGCTCACCT
IFN2 r	CAGCATGGTCCTCTGTAAGGG
OAS1 f	CAAGCTCAAGAGCCTCATCC
OAS1 r	TGGGCTGTGTTGAAATGTGT
OAS2 f	ACAGCTGAAAGCCTTTTGGA
OAS2 r	GCATTAAAGGCAGGAAGCAC
OAS3 f	ATCAAGGTGGTCAAGGAGCG
OAS3 r	TAGACTTGAGAGCTGGGC CT
PKR f	ATGATGGAAAGCGAACAAGG
PKR r	GAG ATGATGCCATCC CGTAG

2.5. Western Blot

Cells were harvested by trypsin, pelleting and subsequent lysis in RIPA lysis buffer (Beyotime Biotechnology, China), and the lysates were then boiled and processed as described in (Yu et al., 2014). Specific antibodies were used to detect different target proteins.

2.6. Nuclear protein extraction

Nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher, USA) according to the manufacturer's protocol. Briefly, cell pellets were collected and subject to CER I, CER II and NER reagents for lysis. To begin with, cell pellets were suspended in CER I and incubated in ice for 10 minutes. Then the pre-chilled CER II was added and vortexed thoroughly. After 1 minute on ice, the tube is centrifuged at maximum speed for 10 minutes. The supernatants

(cytoplasmic extract) were transferred immediately to a clean tube and store at -80 degree. The insoluble pellets remained were suspended in pre-chilled NER and vortexed thoroughly every 10 minutes for 3 times. After centrifugation, the supernatant was also transferred to a clean pre-chilled tube and stored at -80 degree.

2.7. Cytotoxicity assay

Confluent culture of cells were treated with or without caffeic acid for 72 hours. Then, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added 4 hours before the end of culture, and then cells were lysed with 10% SDS, 50% N,N-dimethyl formamide (pH 7.2). OD values were read at 570 nm 6 hours later, and the percent of cell death was calculated(Xu et al., 2014).

2.8. Statistical analysis

Results of experiments were presented as means ± SD. Student's *t*-test was used to analyze the statistical differences using the GraphPad Prism Software (San Diego California, USA). The level of significant difference was set at *p<0.05; **p< 0.01; ***P<0.001.

3. Results

3.1. Caffeic acid inhibited HCV replication

To evaluate the antiviral activity of CA, Huh7.5.1 cells infected with J399EM virus and Con1 cells were treated with different dose of caffeic acid for 72 hours. Results showed that CA markedly inhibited J399EM replication without significantly interfering the viability of cells (Figure1A and 1B). The half maximal inhibitory concentration (IC_{50}) and the half maximal cytotoxic concentration (CC_{50}) was 100±20μM, 1600±30μM respectively. Similar results were obtained in Con1 cells. As shown in Figure 1, HCV RNA（Figure 1C） and protein level（Figure 1D） were notably decreased upon CA treatments.

3.2. Caffeic acid enhanced HO-1 expression

Caffeic acid has long been known for its antioxidant activity (Gulcin, 2006; Migliori et al., 2015). HO-1 is one of the key antioxidant enzymes, and some studies have shown

that the induction of HO-1 could lead to the increase of biliverdin (Lehmann et al., 2010), and biliverdin will trigger IFN-a antiviral response (Lehmann et al., 2010). Therefore, we tried to examine whether CA could modulate HO-1 expression in Con1 cells. A dose-dependent increase of HO-1 RNA expression (Figure 2A) and protein expression (Figure 2B) were detected, which confirmed the induction of HO-1.

3.3. HO-1 is required for Caffeic acid to inhibit HCV replication

To investigate if the antiviral effects of CA is associated with HO-1 induction, Con1 cells were treated with different concentrations of SnPP (an HO-1 inhibitor) in the presence of CA (400μM) for 3 days. Immunoblot assay and qRT-PCR were employed to assess the effects of combination treatments on HCV protein and RNA expression level. HCV RNA （Figure 2C）and protein expression（Figure 2D） was restored after the addition of SnPP and the restored effect was correlated with the concentration of SnPP, although HO-1 expression was not markedly downregulated (Figure 2D). Collectively, these results indicated that CA increased the expression of HO-1 which contributed to CA's antiviral activity.

3.4. Caffeic acid enhanced IFNα related gene expression

It was previously shown that biliverdin, a product of heme degradation by HO-1, could inhibit HCV replication by eliciting the IFNα antiviral response (Zhu et al., 2010). Therefore, efforts were made to investigate whether CA could induce IFNα antiviral response. The results showed that CA treatment increased the mRNA expression levels of IFNα-mediated antiviral genes (Figure 3A), including 2'-5'-oligoadenylate synthetase1 (OAS1), OAS2, OAS3, IFNα2 and PKR (Der et al., 1998). Besides, an increase in OAS1 and PKR protein expression level were also observed (Figure 3B). Collectively, we concluded that CA could trigger the IFNα antiviral response at in mRNA and protein level to inhibit HCV replication.

3.5. Caffeic acid triggered the upregulation and translocation of Nrf2 to increase Ho-1 expression

Next attention was paid to study the pathway modulating HO-1 expression. Nrf2 is one of the upstream transcription factors that can activate HO-1 expression (Alam et al., 1999). To investigate whether Nrf2 is involved in CA-mediated HO-1 upregulation,

the Nrf2 expression was tested in CA-treated Con1 cells. Upon CA treatments, an increasing accumulation of total Nrf2 and nuclear Nrf2 were found in a dose dependent manner (Figure 4A and 4B). When Nrf2 expression was abrogated by Nrf2 siRNA, HO-1 was decreased correspondingly, and the decreased NS5B expression was reversed (Figure 4C). Taken together, the results indicated that CA could induce HO-1 expression via Nrf2 activation.

3.6. Caffeic acid decreased Keap1 expression, in which p62 was involved

To investigate the role of Keap1 in CA-mediated Nrf2 activation (Itoh et al., 1999), CA was used to treat Con1 cells. And a decreased expression of Keap1 was observed in CA-treated Con1 cells (Figure 5A).

P62 is an autophagy substrate that can bind to Keap1, leading to the degradation of Keap1 via selective autophagy (Komatsu et al., 2010; Lau et al., 2010; Taguchi et al., 2012). P62 expression was elevated after the treatment of CA for three days (Figure 5B). In addition, Results show that phosphorylation of p62 (p-p62) was concomitantly enhanced (Figure 5B), which will increase the binding affinity of p62 for Keap1 and further contribute to Keap1 degradation (Ichimura et al., 2013). Along with this, the expression of Cullin3, an adaptor protein responsible for protein ubiquitination showed no marked change (Figure 5B). On the other hand, the induction and activation of LC3, a marker of autophagy was also observed which indicated the involvement of autophagy (Figure 5B). Collectively, CA could increase p62 expression and its phosphorylation and thus enhance Keap1 degradation.

To investigate the role of p62 phosphorylation on Keap1 degradation (Ichimura et al., 2013), rapamycin, an mTORC1 kinase inhibitor, was used to treat Con1 cells in the presence of CA. Upon combinational treatment, p62 and p-p62 expression were abolished dose-dependently and the decreased expression of Keap1 was also slightly restored (Figure 6A). And the proteasome inhibitor, MG132 was also used to investigate the role of the proteasome in CA-mediated Keap1 degradation. Results showed that Keap1 remained unchanged upon MG132 treatments in the presence of CA (Figure 6B), which confirmed that Keap1 degradation was not due to the proteasome degradation. In addition, p62 siRNA was used to knock down p62

expression. Consistent with rapamycin treatments, Keap1 expression was also restored, which indicated the involvement of p62 in CA-mediated Keap1 degradation (Figure 6C). Taken together, we concluded that p62 was required for Keap1 degradation.

4. Discussion

In this study, we showed that CA could inhibit HCV replication in HCV replicon (1b) and infectious J399EM system (Figure 1) (Han et al., 2009; Lohmann et al., 1999) by increasing HO-1 expression (Figure 2), which led to the downstream activation of IFNα antiviral response (Figure 3). Following that, we further uncovered CA could activate Nrf2 to induce HO-1 expression (Figure 4). When cells were transfected with Nrf2 siRNA, HO-1 expression was notably abrogated (Figure 4) and the CA-mediated downregulation of HCV protein was restored, suggesting HO-1 was induced by Nrf2 and required for CA's antiviral effects. In line with Pang et al. (Pang et al., 2016), we also found that CA could decrease Keap1 expression (Figure 5), and this will subsequently contribute to the release and accumulation of Nrf2 and its translocation from the cytoplasm into nucleus to induce related gene expression. Moreover, the accumulation of p62, an adaptor protein responsible for Keap1 degradation was also observed after the treatment of CA (Figure 5). Along with this, the phosphorylated p62 was also upregulated. Due to the involvement of mTORC1 in p62 phosphorylation (Ichimura et al., 2013), rapamycin, an mTORC1 inhibitor was used to investigate whether it could interrupt p62 phosphorylation. The Keap1 expression was partly but not completely reversed upon rapamycin treatments, possibly because rapamycin is both an autophagy inducer and mTORC1 inhibitor (Li et al., 2013). The rapamycin's inhibitory effects on p62 phosphorylation and then Keap1 degradation was partially counteracted by the enhanced autophagy caused by rapamycin. p-p62 and p62 expression decreased when cells were treated with rapamycin in the presence of CA (Figure 6A)，possibly because p62 is a downstream target of the transcription factor Nrf2 (Jain et al., 2010). The inhibition of p62 phosphorylation had led to the stabilization of Keap1 and then the proteasomal

degradation of the Nrf2, and thus the downregulation of p62. Similar results were also obtained using siRNA of p62 (Figure 6C). The knockdown of p62 led to less binding of p62 for Keap1, thus more Keap1 was stabilized. More Keap1 retained in cytoplasm, more proteasomal degradation of the Nrf2. Therefore, less Nrf2 will translocate into the nucleus to induce p62 expression. To exclude the possibility that Keap1 might be degraded via the proteasome pathway, a proteasome inhibitor, MG132 was employed to investigate the role of the proteasome in Keap1 degradation. We found that Keap1 remained unchanged upon MG132 treatments in the presence of CA (Figure 6B). The proposed model of the mechanism of CA's anti-HCV effect has been presented in Figure 7.

Prevalence of oxidative stress during HCV replication is widely observed (Ivanov et al., 2013; Wang and Weinman, 2013). It was shown that most of the HCV-related proteins, including nonstructural and structural proteins, were involved in the induction of oxidative stress (Ivanov et al., 2011; Pal et al., 2010), and oxidative stress has been long considered as a choice of anti-HCV strategy. The Keap1/Nrf2 signaling pathway is one of the important antioxidant pathways that can directly regulate the expression of a series of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase, NADPH : quinone oxidoreductase (NQO1), HO-1 (Tripathi and Jena, 2010). CA has been well known for its antioxidant effects (Gulcin, 2006; Migliori et al., 2015). We found that CA could trigger HO-1 expression via Keap1/Nrf2 pathway. As for the reason why CA can activate the Keap1/Nrf2 signaling pathway, it can possibly be explained by that CA is a kind of compound possessing electrophilic carboxyl group. Upon exposure to CA, carboxyl group of CA reacts with cysteine sulfhydryl groups of Keap1 leading to the change of Keap1 conformation and the dissociation of Keap1 from Nrf2, and then activation of Nrf2 (Dinkova-Kostova et al., 2002; Eggler et al., 2005; Holland and Fishbein, 2010; Marino and Gladyshev, 2012). Then the released Keap1 was degraded by the p62-mediated selective autophagy (Ichimura et al., 2013; Taguchi et al., 2012).

In addition to Keap1, there still exist several factors that are involved in regulating Nrf2 activity, including phosphorylation of Nrf2 by various protein kinases, like

protein kinase C (PKC)(Huang et al., 2002), phosphatidylinositol 3-kinase (PI3K/Akt), GSK-3β(Rada et al., 2011), c-Jun N-terminal kinase (JNK)(Yuan et al., 2006), extracellular signal-regulated kinase (ERK) (Yuan et al., 2006). Besides, Protein partners (p21)(Chen et al., 2009), caveolin-1(Zheng et al., 2012) and epigenetic factors (micro-RNAs)(Sangokoya et al., 2010; Yang et al., 2011) also play a role in the regulation of Nrf2 activity. Because biliverdin can exert anti-HCV effects through impairing HCV NS3/4A proteinase activity (Zhu et al., 2010) CA could possibly inhibit HCV NS3/4A proteinase activity. Further work is needed to elucidate the role of these factors in CA's antiviral effects. The uncover of mechanism of CA pharmacological effects may encourage a wider application of CA due to its diverse pharmacological effects and may also lay the foundation for the further optimization of CA structure.

Taken together, our results showed that CA could inhibit HCV replication, which was mediated by the induction of HO-1 through Keap1/Nrf2 pathway. The increased expression of HO-1 will lead to the activation of IFNα antiviral response and thus block HCV replication.

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6. Conflicts of interests

We declared no conflicts of interest concerning the authorship of this piece of paper.

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8. Figures and legends

Figure 1. CA inhibited HCV replication. Antiviral effects of CA were evaluated in infectious J399EM (2a) (A, B) and HCV replicon system Con1 (1b) cells (C, D). (A) Cytotoxicity of CA on Huh7.5.1 cells in the presence or absence of CA. Viability was determined with MTT assays, and presented as percentage of that of control cells. (B) The inhibitory effects of CA on infectious HCV system. Huh7.5.1 cells were infected with J399EM at a MOI of 0.1 and six hours later, the viral supernatants were replaced with various doses of CA and incubated for 72 hours. Then the fluorescence (OD value) was read out in a microplate reader. The OD value of control cells were considered as 100%. (C, D) HCV RNA and protein level were decreased by CA in Con1 cells. Total cellular RNA and protein was extracted and subject to qRT-PCR and Western Blot, respectively. The absolute number of HCV RNA copies were detected and normalized to the internal control GAPDH. The NS5B and β-actin protein expression level was detected using specific antibodies.

Figure 2. CA induced HO-1 expression to inhibit HCV replication. (A, B) HO-1 RNA and protein level was increased significantly upon CA treatments. The Con1 cells were treated with different concentrations of CA for three days, then the cells were collected, RNA and protein extracted and subject to RT-qPCR and Western Blot respectively. The absolute number of HO-1 RNA copies were detected and normalized to the internal control GAPDH. The β-actin expression was used as protein loading control. HCV RNA（C）and protein level（D） was restored by

treatment of SnPP, an HO-1 inhibitor in the presence of CA. The HCV RNA level was normalized to the internal control GAPDH and described as percentage of that of CA-untreated cells (control, considered as 100%). Data were presented as the means of normalized data ± standard deviations (error bars) based on at least three independent experiments. *p<0.05; ** p <0.01.

Figure 3. CA triggered IFNα antiviral response. (A, B) CA induced IFNα related gene expression at mRNA and protein levels. Con1 cells were treated with indicated concentrations of CA for three days. The mRNA levels of specific genes were normalized to the internal control GAPDH and presented as percentage of that of CA-untreated cells. The PKR and OAS1 antibodies were used to evaluate their expression level. β-actin was used as a loading control.

* p <0.05; **, p <0.01; *** p <0.001.

Figure 4. CA activated Nrf2 to induce HO-1 expression. (A, B) CA increased Nrf2 expression and led to Nrf2 accumulation in nucleus. N-Nrf2, Nrf2 in Nucleus; Cyto-Nrf2, Nrf2 in cytoplasm. (C) Nrf2 was required for CA-mediated HO-1 induction. The Con1 cells were treated with various concentrations of CA for three days. The total cellular protein and nuclear and cytoplasmic protein was subsequently extracted respectively. Antibodies for Nrf2, β-actin, LaminB, HO-1 were used to detect specific protein expression.

Figure 5. CA downregulated Keap1 expression, in which p62 was involved. (A) CA decreased Keap1 expression in a dose-dependent way. (B) CA increased p62 to downregulate Keap1. Con1 cells were treated with indicated concentrations of CA for three days. Antibodies for Keap1, p62, Cullin3, LC3 were used to assess the protein expression level.

Figure 6. The role of p62 in CA-mediated downregulation of Keap1. (A) Rapamycin (rapa), an mTORC1 inhibitor restored the decreased Keap1 caused by CA; (B) MG132, a proteinase inhibitor, didn't reverse the decreased expression of Keap1. (C) p62 was required for CA-mediated Keap1 downregulation. Con1 cells were treated with CA with or without rapamycin, MG132, p62 siRNA (Si-p62) for three days. Antibodies for Keap1, p62, p-p62, β-actin were used to assess the protein expression level.

Figure 7. Scheme of the mechanism of CA's antiviral effects. To begin with, CA leads to the induction of p62 and its phosphorylation, which contributes to Keap1's degradation by p62-mediated autophagy. Then the decrease of Keap1 will disrupt the ubiquitination of Nrf2 and encourage its stabilization. The stabilized Nrf2 will accumulate in the cytoplasm and translocate into the nucleus to activate the related gene expression, including HO-1, and the induced HO-1 will lead to activation of the IFNα antiviral response and inhibition of HCV replication.

Figure 1

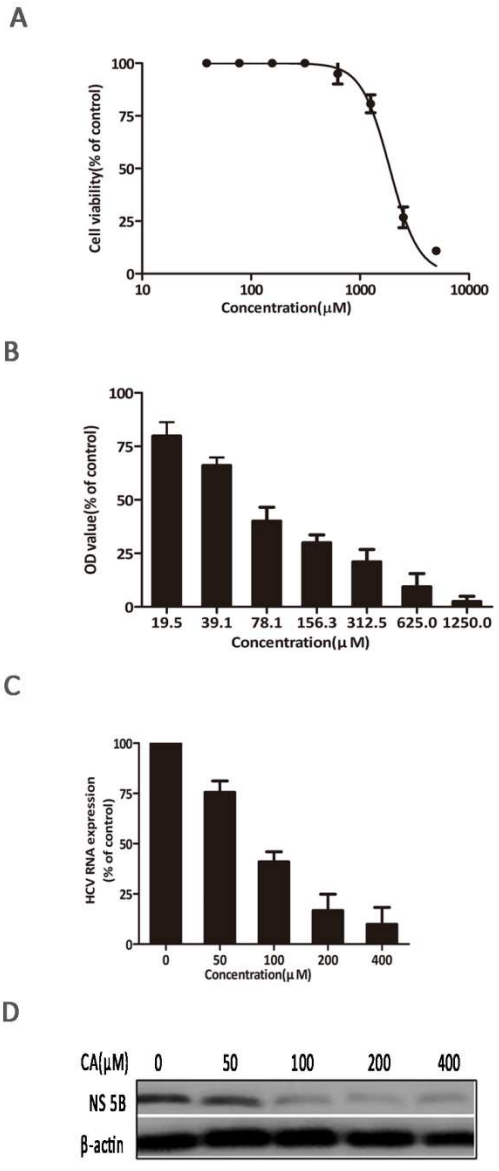


Figure 2

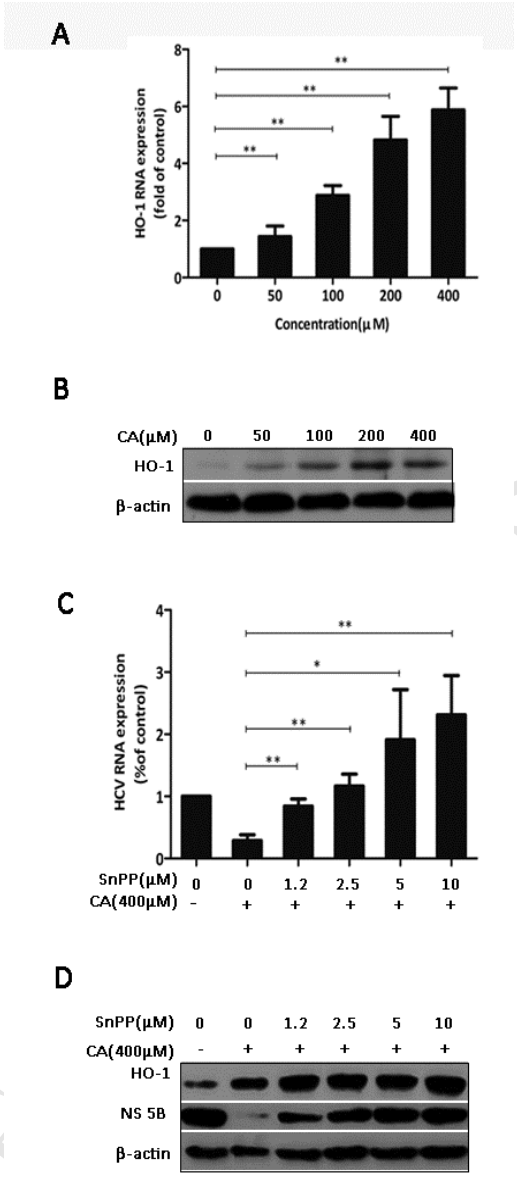

A
HO-1 RNA expression (fold of control)
Concentration(μM)
B
CA(μM) 0 50 100 200 400
HO-1
β-actin
C
HCV RNA expression (%of control)
SnPP(μM) 0 0 1.2 2.5 5 10
CA(400μM) - + + + + +
D
SnPP(μM) 0 0 1.2 2.5 5 10
CA(400μM) - + + + + +
HO-1
NS 5B
β-actin

Figure 3

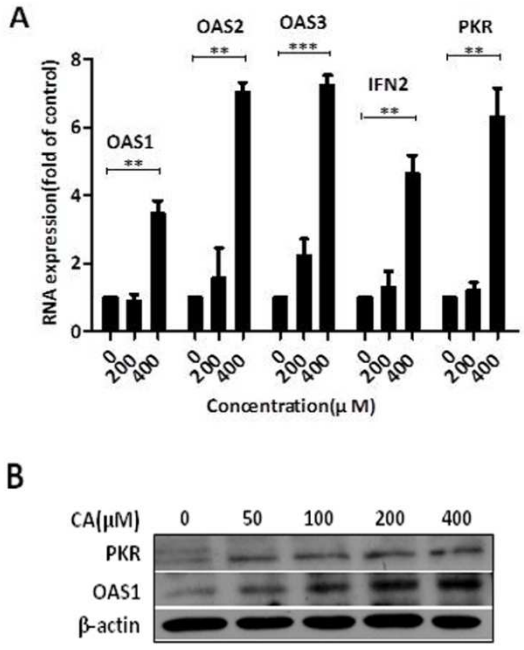


Figure 4

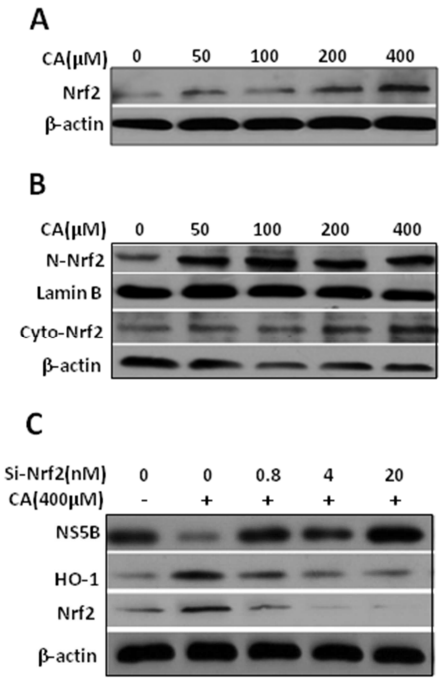


Figure 5

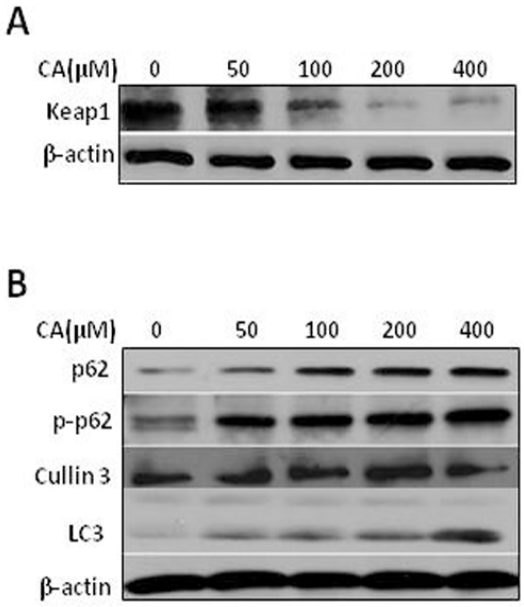


Figure 6

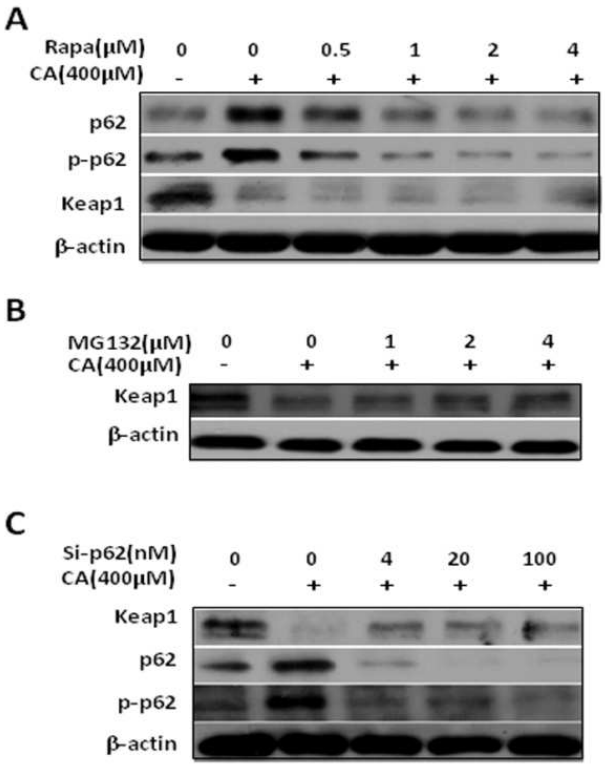
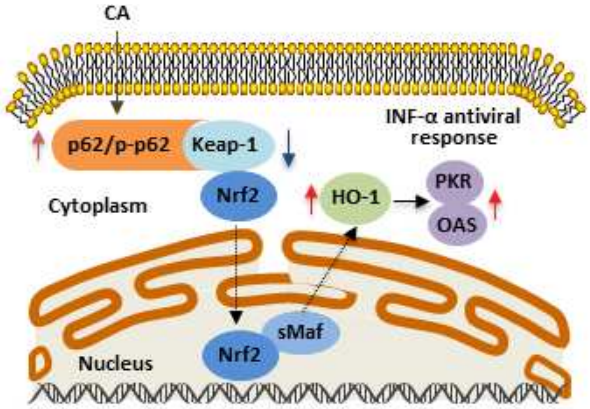


Figure 7



Highlights:

- CA inhibits HCV replication by inducing HO-1 expression and thus eliciting IFNα antivral response
- CA activates Nrf2 to induce HO-1 expression
- CA increases p62 expression and thus downregulates Keap1 expression to activate Nrf2
- Lay the foundation for a wider application of CA and the potential optimization of CA structure