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ORIGINAL ARTICLE

Basic Study

Anti-hepatitis C virus potency of a new autophagy inhibitor using human liver slices model

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Abstract

AIM: To evaluate the antiviral potency of a new anti-hepatitis C virus (HCV) antiviral agent targeting the cellular autophagy machinery.

METHODS: Non-infected liver slices, obtained from human liver resection and cut in 350 μm-thick slices (2.7×10^6 cells per slice) were infected with cell culture-grown HCV Con1b/C3 supernatant (multiplicity of infection = 0.1) cultivated for up to ten days. HCV infected slices were treated at day 4 post-infection with GNS-396 for 6 d at different concentrations. HCV replication was evaluated by strand-specific real-time quantitative reverse transcription - polymerase chain reaction. The infectivity titers of supernatants were evaluated by foci formation upon inoculation into naive Huh-7.5.1 cells. The cytotoxic effect of the drugs was evaluated by lactate dehydrogenase leakage assays.

RESULTS: The antiviral efficacy of a new antiviral drug, GNS-396, an autophagy inhibitor, on HCV infection of adult human liver slices was evidenced in a dose-dependent manner. At day 6 post-treatment, GNS-396 EC50 was 158 nmol/L without cytotoxic effect (compared to hydroxychloroquine EC50 = 1.17 μmol/L).

CONCLUSION: Our results demonstrated that our *ex vivo* model is efficient for evaluation the potency of autophagy inhibitors, in particular a new quinoline derivative GNS-396 as antiviral could inhibit HCV infection in a dose-dependent manner without cytotoxic effect.

Key words: Host antiviral therapy; Hepatitis C virus; Tissue culture; Autophagy; Quinoline derivative

Core tip: Hepatitis C virus (HCV) infection (or spread) is a serious public health challenge counting approximately 170 million people that are chronically infected worldwide. Efficient interferon-free treatments with new direct acting antivirals are expected to cure more than 90% of HCV-infected patients but they are not available in all the countries. Autophagy machinery is required to initiate HCV replication. Host antiviral therapy is an additional option for the treatment of HCV infection. The new autophagy inhibitor GNS-396 demonstrated significant efficacy and additive activity in inhibiting HCV replication and might be an additional option to treat HCV infected individuals.

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INTRODUCTION

Approximately 170 million people worldwide are chronically infected with hepatitis C virus (HCV)[1]. Until recently, the most effective treatment against HCV infection was the combination of pegylated interferon-α 2a or b and ribavirin (PR) which achieved sustained virological response (SVR) in about 45% of individuals infected by HCV genotype 1, 65% by HCV genotype 4, 70% by HCV genotype 3 and more than 85% by HCV genotype 2[2,3]. The frequent side effects associated with PR and the rates of non response to PR includes partial or null virologic response and breakthrough or relapse after PR discontinuation. Thus, development of novel and more effective antiviral treatments were essential[4].

Two HCV NS3 protease inhibitors (PI), boceprevir (BOC) and telaprevir (TVR) have been approved and combined with PR, have increased the SVR to about 75% in therapy naïve HCV genotype 1 infected patients[5-9]. Over the past few years, other direct acting antivirals (DAAs) were developed[10-14] as second generation of PI with higher antiviral potency, HCV NS5A replication complex inhibitors and nucleotide analogue HCV NS5B polymerase inhibitors[13] as well as host-targeted indirect antivirals like cyclophilin inhibitors[15] and lambda interferon[15]. Interferon-free treatments with new DAAs are expected to cure more than 90% of HCV-infected patients[16]. But they are not available in all the countries[17]. At the present time, triple therapy combining PR with NS3 PI (TVR or BOC) is going to remain the main treatment for HCV patients[16-21]. That is why it appears important to continue research in limiting virus replication and the autophagy inhibition could be a new additional pathway because of recent evidences obtained regarding to an increased autophagic response in the liver of chronically HCV infected patients[22].

Autophagy is a catabolic process which degrades a cellular own component through the lysosomal machinery[23]. It has been shown that autophagy is activated during virus and bacterial infection[24] and that some viruses can use the autophagy system to facilitate their own replication[25-29]. Previously, several studies evidenced that HCV infection resulted in endoplasmic reticulum stress and autophagy responses, that HCV regulated the autophagy pathway, that the autophagy machinery was required to initiate HCV replication, and finally, that the suppression of autophagy inhibited HCV replication[30-35]. Interestingly, it has been demonstrated that HCV induces autophagosomes *via* a Class Ⅲ PI3K-independent pathway and uses autophagosomal membranes as sites for its RNA replication[36].

The lysosomotropic anti-malarial drugs, chloroquine (CQ) and hydroxychloroquine (HCQ), belonging to the quinoline family, are among the autophagy inhibitors, which act by preventing the acidification of lysosomes, leading to the inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation[23]. In fact, CQ exerts an inhibitory effect for several RNA viruses including coronaviruses, flaviviruses and human

immunodeficiency virus[37-39]. Recently, it has been shown that a treatment with CQ of HCV infected cells suppressed the replication of the virus in a dose-dependent manner by preventing the autophagic proteolysis[40].

In the present study, we used the established *ex vivo* model of primary human liver slices culture which allows to the *de novo* replication of primary viral isolates and production of high titer infectious HCV particles[41] to evaluate the potential antiviral potency GNS-396[42], a new autophagy inhibitor in comparison with a well-known autophagy inhibitor, HCQ. Presented results might be additional options to treat HCV infected individuals.

MATERIALS AND METHODS

Human liver tissue specimens

Adult human primary liver tissue samples were obtained from HCV and also hepatitis B virus, and human immunodeficiency virus seronegative patients who underwent liver resection surgery, mainly for liver metastasis in the absence of underlying liver disease. Experimental procedures were carried out in accordance with French laws and Regulations.

Liver slices preparation, culture and infection

Slices were prepared and cultured as described[41,43]. Briefly, uninfected human liver slices, obtained from human liver resection, were cut into 350 μm thick slices of (2.7×10^6 cells per slice) with a vibratome (Leica, VTS1200) and transferred to 0.4 μm organotypic culture inserts (Millipore) in 12-wells plates (1 slice/well) containing 2 mL of complete Dulbecco's modified eagles's medium (DMEM) culture media and maintained at 37 ℃ under a constant flow of humidified 95% O_2/5% CO_2 for up to 24 h before viral infection. Cell number for tissue slices was estimated at approximately 2.7×10^6 cells per slice based on a 14-cell thick slice (cell diameter approximately 25 μm)[41]. The complete culture medium consisted of DMEM with 4.5 g/L D-glucose and glutamine (Life Technologies) supplemented with 10% fetal calf serum (Life Technologies, 16000-044), 5% penicillin-streptomycin (Life Technologies, 10378-016), 1% amphotericin (Sigma Aldrich), 5 μg/mL insulin (Life Technologies, 51500-056), 0.4 μg/mL dexamethasone (Sigma Aldrich, D4902), 10 mmol/L HEPES (Life Technologies, 15630080), non-essential amino acids (Life Technologies), 20 mmol/Lsodium pyruvate (Life Technologies) and 50 μg/mL ascorbic acid (Sigma Aldrich). One day post-culture in twelve-transwell plates, human primary liver slices were inoculated with HCV Con1/C3 at a multiplicity of infection equal to 0.1 at 37 ℃ in the same culture conditions as described above, for overnight. The infectious clone Con1/C3 (genotype 1b) (JFH1-derived chimeric viruses whose structural proteins are encoded by the genotype 1b-HCV sequence Con1)[44] could efficiently infect human liver slices which maintain their hepatocyte differentiation and retain normal physiological and biochemical parameters for at least 10 d. The inoculum was then removed; the slices were washed three times with PBS and then supplemented with complete culture medium. Then, liver slices were cultured without medium replacements, as previously described[41].

HCV RNA transfection and virus production

To produce HCVcc, viral RNAs were transcribed *in vitro* and electroporated into Huh-7.5.1 cell line (kindly provided by Professor Francis V Chisari, The Scripps Research Institute, La Jolla, CA), as described previously[45]. The infectious titer of cell culture supernatants was evaluated by classical titration assay[45]. In brief, the HCV infection of Huh-7.5.1 cells was performed with serial 10-fold dilution of viral supernatants. Seventy-two hours later, the formation of infected cells foci were detected by staining with human HCV positive sera or mouse monoclonal antibodies directed against HCV core (Ozyme) and nonstructural (NS5A) (Virostat, clone1877) proteins. Titrations were performed in duplicate.

Quantification of HCV strands RNA by real-time quantitative reverse transcription- polymerase chain reaction

A strand-specific real-time quantitative reverse transcription-polymerase chain reaction technique to quantify the intracellular levels of positive and negative strand HCV RNA was performed as previously described[46-49]. The quantification of 28S rRNA was used as an internal standard to quantify HCV in total liver RNA, as previously described[46], (threshold of detection: 25 copies/reaction). Briefly, reverse transcription was carried out using oligo(dT) primer (Life Technologies) and Moloney murine leukemia virus reverse transcriptase (Promega) as recommended by the manufacturer. Real-time polymerase chain reactions were performed using the Light CyclerR (Roche Applied Science) and Fast Start DNA Master SYBR Green Ⅰ kit (Life Science, Roche) according with the manufacturer's protocol.

Reverse transcription was performed using primers located in the 5' NCR region of HCV genome, tag-RC1 (5'-GGC CGT CAT GGT GGC GAA TAA GTC TAG CCA TGG CGT TAG TA-3')[47] and RC21 (5'-CTC CCG GGG CAC TCG CAA GC-3')[48] for the negative and positive strands, respectively, as described previously[46]. After a denaturation step performed at 70 ℃ for 8 min, the RNA template was incubated at 48 ℃ for 5 min in the presence of 200 ng of tag-RC1 primer and 1.25 mmol/L of each deoxynucleoside triphosphate (dNTP) (Promega) in a total volume of 12 μL. Reverse transcription was carried out for 60 min at 60 ℃ in the presence of 20 U RNaseOutTM (Life Technologies) and 7.5 U ThermoscriptTM reverse transcriptase (Life Technologies), in the buffer recommended by the manufacturer. An additional treatment was applied by adding 1 μL (2U) RNaseH (Life Technologies) for 20 min at 37 ℃. The first round of nested PCR was performed with 2 μL of the cDNA obtained in a total volume of 50 μL, containing 3 U Taq polymerase (Promega), 0.5 mmol/L dNTP Mix (Promega), and 0.5 μmol/L RC1 (5'-GTC TAG CCA TGG CGT TAG TA-3')

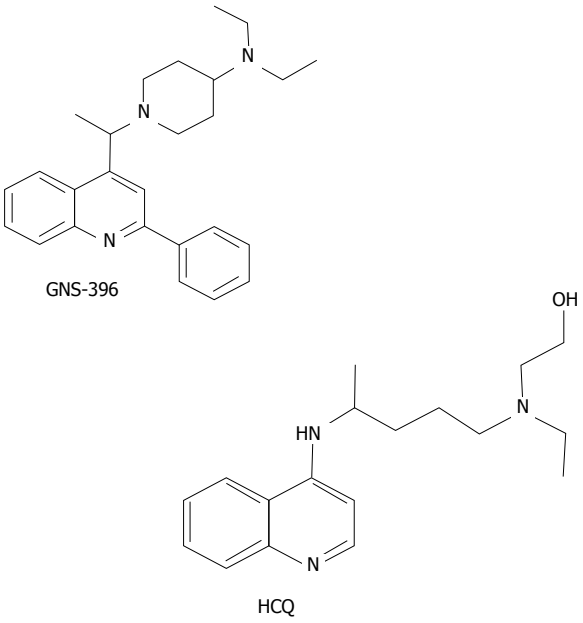

Figure 1 GNS-396 and hydroxychloroquine structures. HCQ: Hydroxychloroquine.

and RC21 primers for positive-strand amplification, or tag (5′-GGC CGT CAT GGTGGC GAA TAA-3′) and RC21 primers for negative strand amplification. The PCR protocol consisted of 18 cycles of denaturation (94 ℃ for 1 min), annealing (55 ℃ for 45 s), and extension (72 ℃ for 2 min). The cDNA obtained was purified using the Quick-clean kit (Qiagen), according to the manufacturer's instructions, and 2 μL of the purified product suspended in 10 μL nuclease free water (Promega) were then subjected to real-time PCR. The reaction was carried out using the DNA Fast Start SYBR Green Kit (Life Science, Roche), with LightcyclerTM instruments and technology (Roche Diagnostics). PCR amplifications were performed in a total volume of 20 μL, containing 3 mmol/L $MgCl_2$, 2 μL DNA Master green (Life Science) and 50 ng of the 197 R (5′-CTTTCGCGACCCAACACTAC-3′) and 104 (5′-AGAGCCATAGTGGTCTGCGG-3′) primers[48,49]. The PCR protocol consisted of one step of initial denaturation for 10 min at 94 ℃, followed by 40 cycles of denaturation (95 ℃ for 15 s), annealing (57 ℃ for 5 s), and extension (72 ℃ for 8 s). After amplification, the specificity of PCR products was checking by a melting curve analysis.

Western blotting

Western blotting was performed as following. Each liver slice was washed 3 times in PBS, incubated in Laemmli buffer[50] at 100 ℃ for 10 min. The lysate was passed through a 26 G needle, 10 times and kept at -80 ℃. Before electrophoresis in pre-cast sodium dodecyl sulfate polyacrylamide gel 4%-12% (Life Technologies), the samples were incubated at 95 ℃ for 5 min. After electrophoresis, proteins were transferred to a 0.22 μm Protran membrane BA83 (Schleicher and Schuell) and HCV proteins were detected by Western blotting using mouse monoclonal antibodies to core (C7-50, 1:10000) (Ozyme), to NS5A (1:2000) (Virostat, clone 1877), to LC3 proteins (Sigma-Aldrich) and to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam), and to β-actin (Pierce biotechnology). Horseradish peroxidase-conjugated anti-mouse IgG (GeHealthCare Life Sciences) at the dilution of 1:50000 were used as secondary antibodies. The reactions were developed using enhanced chemiluminescence detection reagents (GeHealthCare Life Sciences), followed by exposure to X-OMAT film (GeHealthCare Life Sciences). LC3-Ⅱ protein expression analysis was performed with Image J software.

Drugs inhibition of HCVcc Con1/C3 replication and cytotoxicity assays

The HCVcc Con1/C3 inhibition either by pegylated-interferon α-2a (peg-INF) (Roche, Pegasys) or/and ribavirin (RBV) (Schering Plough, Rebetol) or TVR (Janssen-Cilag, Incivo) or BOC (Schering-Plough, Boceprevir) or SOF (Gilead Sciences Intl Ltd, Sofosbuvir) or GNS-396 (Figure 1) (Genoscience Pharma, Marseille, France) or HCQ (Figure 1) (Genoscience Pharma, Marseille, France) or 0.5% dimethylsufoxide (DMSO) (Sigma Aldrich) as a carrier control, and the cytotoxicity assays were performed as following. At day 4 post-infection with HCVcc Con1/C3 the human liver slices were treated by addition of different concentrations of the following drugs: peg-INF or/and RBV or TVR or BOC or SOF or HCQ (0.1, 1, 2.5, 5 μmol/L or a new quinoline derivative, GNS-396 (0.1, 1, 2.5, 5 μmol/L) alone or 0.5% DMSO as a carrier control, to culture medium, twice daily, up to day 10 post-infection. The infectivity (ffu/mL) was measured at day 2, day 4 or day 6 post-treatment depending on the experiment as described[41]. All the experiments were performed in triplicate. The cytoTox 96R Non-Radioactive Cytotoxicity Assay (Promega, G1780) was used to assess the potential cytotoxicity of the drugs. Results of lactate deshydrogenase (LDH) leakage were compared to the carrier control calculated (Figure 2) as described previously[51].

Evaluation of autophagy modulation and inhibition

Autophagy modulation was evaluated on HeLa cells treated with GNS-396, a new quinolone derivative. For tracking different stages of autophagy the tandem fusion of mRFP and EGFP fused to LC3 make a pH-sensitive sensor (mRFP-EGFP-LC3) that is used to monitor autophagy in live cells[52]. The EGFP tag is acid-sensitive while the mRFP tag is not. The double tagged LC3 can be used to label autophagosomes, amphisomes and autolysosomes. In autophagosomes, both tags emit yellow light. However, the fusion of autophagosomes to acidic lysosomes results in acidic autolysosomes where the green fluorescence from GFP is lost. Subsequently, the red fluorescence from mRFP is lost when the double tagged protein is degraded. The autophagic flux inhibition was shown using a SkBr3 mRFP1-EGFP-LC3 stable breast cancer cell line treated with 100 μmol/L GNS-396 or 100 μmol/L HCQ during 6 h. HCQ was used as a positive control of autophagy inhibition. Cell images were obtained using an epifluorescence microscope (Nikon,

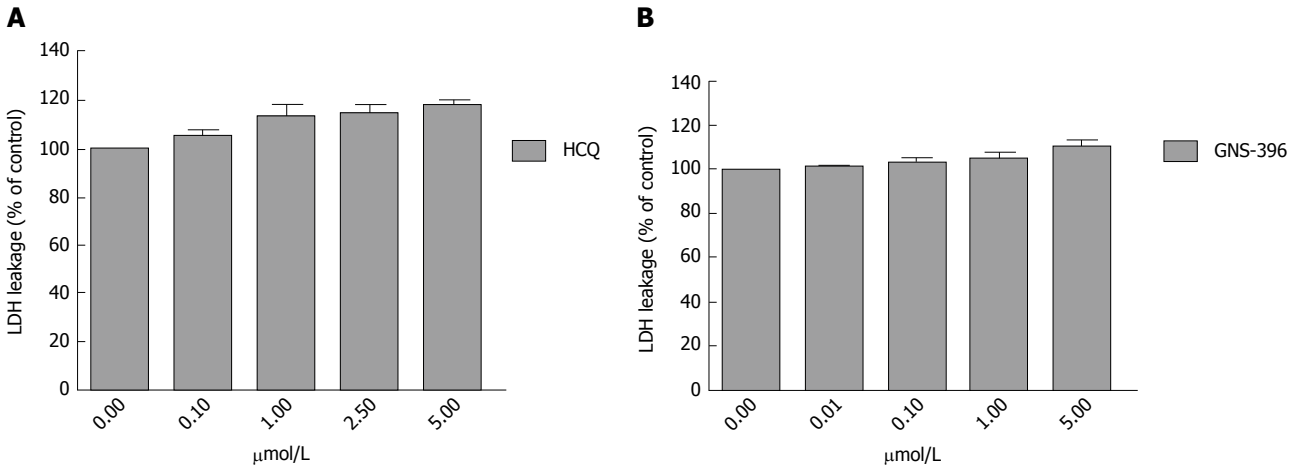

Figure 2 Cytotoxicity assays of the drugs used hydroxychloroquine (A) and GNS-396 (B). Percentages of lactate deshydrogenase (LDH) leakage are relative to DMSO control-treated liver slices. Drugs cytotoxicity is significant at % of control > 120. All experiments were performed on triplicate. Values are expressed as means ± SE, comparisons were performed using the Mann-Whitney rank-sum test ($P < 0.001$). HCQ: Hydroxychloroquine; DMSO: Dimethylsufoxide.

Eclipse Ci).

Autophagy inhibition was evaluated on HeLa cells treated with GNS-396, at different drug concentrations (1, 10, 100 μmol/L) during 4 h or 6 h in the presence or absence of bafilomycine A1 (100 nmol/L) (Sigma-Aldrich) added for the last 2 h. Bafilomycin A1 (BafA1) is an autophagy inhibitor as endosomal acidification inhibitor. It is a known inhibitor of the late phase of autophagy. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes. Bafilomycin A1 acts by inhibiting vacuolar H+ ATPase[53,54]. HCQ was used as a positive control of autophagy inhibition. The LC3-Ⅱ protein expression in cell lysates was evaluated by Western-blot assay [anti-LC3 antibody (Sigma-Aldrich)] compared to either GAPDH protein expression [anti-GAPDH antibody (Abcam)] or β-actin protein expression [anti-actin antibody (Pierce biotechnology). LC3-Ⅱ protein expression analysis was performed with Image J software.

Statistical analysis

At different days of the kinetics, the results were obtained from the mean of the three slices culture. Statistical tests were performed using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, United States). Values are expressed as means ± standard errors of the mean. The data were compared using an unpaired two-tailed student's *t*-test or the Mann-Whitney rank-sum test. *P*-value < 0.05 was considered significant.

RESULTS

Validation of GNS-396, a new quinoline derivative, as inhibitor of autophagy

We evaluated the effect of GNS-396 (Figure 1)[42], a new quinoline derivative, on autophagy by treatment of HeLa cells with various concentrations of GNS-396 during 6 h. HCQ was used as a positive control of autophagy inhibition. The microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble ubiquitin-like protein with a molecular mass of approximately 17 kDa that exists ubiquitously in mammalian tissues and cultured cells, as an unconjugated form (LC3-Ⅰ) or conjugated to autophagosomes membranes (LC3-Ⅱ: lipidated form). During autophagy, a cytosolic form of LC3 (LC3-Ⅰ) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-Ⅱ), which is recruited to autophagosomal membranes allowing for the closure of the autophagic vacuole. Autophagosomes fuse with lysosomes to form autolysosomes, and intra-autophagosomal components are degraded by lysosomal hydrolases. At the same time, LC3-Ⅱ in autolysosomal lumen is degraded. Thus, lysosomal turnover of the autophagosomal marker LC3-Ⅱ reflects autophagic activity. Analysis of LC3 intracellular expression by Western blotting demonstrated an increase of normalized LC3-Ⅱ protein expression when HeLa cells were treated with GNS-396, in a dose-dependent manner (Figure 3A), reflecting the accumulation of autophagosomes in cells, and therefore an effective modulation of the autophagy. Consequently, GNS-396 is a dose-dependent autophagy modulator with a magnitude of normalized LC-3 Ⅱ similar to which achieved with HCQ treatment, a well-known autophagic inhibitor (Figure 3B). Similar results were obtained on Huh7.5.1 cell line (data not shown).

To evaluate if the observed accumulation of autophagosomes after GNS-396 treatment was a consequence of either a stimulated production of new autophagosomes (in this case, GNS-396 would be an autophagy inducer) or a result of autophagosome clearance blockage (in this case, GNS-396 would be an autophagy inhibitor), HeLa cells were treated with different concentrations of GNS-396 in the absence or presence of a lysosomal protease inhibitor, Bafilomycin A1, that increases lysosomal pH and blocks autophagosome-lysosome fusion (Figure 3C) and LC3 protein levels were measured. HCQ was used as a positive control of autophagy inhibition (Figure 3D). After 4 h exposure of HeLa cells to GNS-396 (100 μmol/L),

the accumulation of LC3-Ⅱ was observed (Figure 3C) which was not enhanced in the presence of BafA1, supporting the idea that GNS-396 inhibits autophagic flux as HCQ (Figure 3D). To confirm that GNS-396 is an autophagy inhibitor, the autophagic flux was monitored by fluorescence microscopy, using the mRFP-EGFP-LC3 tandem-tagged fluorescent protein in SkBr3 mRFP-EGFP-LC3 stable cell line (Figure 4). In green/red merged images, yellow dots (*i.e.*, mRFP+EGFP+) indicate autophagosomes or non-acidic autolysosomes, while red dots (*i.e.*, mRFP+EGFP-) indicate autolysosomes. The autophagy flux is increased when both yellow and red punctua (dots) are increased in cells while the autophagic flux is blocked when only yellow punctua (dots) are increased without an accompanying increase of red punctua in cells. SkBr3 mRFP-EGFP-LC3 stable cell line was treated during 6 h with either GNS-396 (Figure 4C) or HCQ, a well-known autophagic inhibitor (Figure 4B) (100 μmol/L). An accumulation of yellow punctua (dots) corresponding to autophagosomes or non-acidic autolysosomes was noticeable (Figure 4B and C), indicating that GNS-396 blocks the autophagic flux, and may act as lysosomotropic agent as HCQ.

Modulation of autophagy and inhibition of HCV infection in human liver slices model by GNS-396 treatment

The level of LC3 and viral proteins expression were analysed by Western blotting after 1, 4, 6 and 10 d post-infection (Figure 5). HCV infection induced autophagy with an increase of protein LC3-Ⅱ expression (Figure 5B) as compared to non-infected liver slices (Figure 5A), along with an increase of intracellular expression of the core and NS5A proteins consistent with the previous reports[22,36]. Intracellular expression of the viral proteins was decreased significantly at day 6 post-treatment with HCQ (1 μmol/L) or GNS-396 (1 μmol/L) (Figure 5D) in comparison with HCVcc infected liver slices not treated (Figure 5B). The HCQ- and GNS-396-treatment induced an accumulation of LC3-Ⅱ protein in HCV infected liver slices treated with 1 μmol/L HCQ or 1 μmol/L GNS-396 (Figure 5D) in comparison either with not infected liver slices treated (Figure 5C) or not (Figure 5A), or with HCV infected liver slices without treatment (Figure 5B). At day 10, the normalized LC3-Ⅱ protein expression increased when liver slices infected (Figure 5D) or not (Figure 5C) were treated either with GNS-396 (1 μmol/L) or HCQ (1 μmol/L). The GNS-396 and HCQ effects were tested on the *de novo* viral production of HCVcc Con1 infected liver slices (Figures 6 and 7). At day 4 post-infection, HCVcc Con1 infected liver slices were treated for 6 d with different concentrations either of GNS-396 or HCQ. From day 1 to day 6 post-treatment, the HCV RNA replication (Figure 6A and B) and the infectivity (Figure 7A and B) were inhibited in a dose-dependent manner. The addition of RBV with the new drug GNS-396 showed no significant difference in the viral inhibition (data not shown).

EC50 analysis of HCV replication with GNS-396 treatment compared to that of validated antiviral drugs

The ability of various concentrations of different antiviral drugs to inhibit HCV replication was measured by detecting the replication of negative strands HCV RNA (Figure 6A and C) (Table 1). The calculated EC50 of different antiviral drugs is listed as Table 1 and compared to GNS-396. In summary, our model confirms that the antiviral activity of triple therapy was higher than that of the dual therapy by PR as extensively reported in clinical trials[5,6]. The new quinoline derivative GNS-396 has about 10-fold lower EC50 than HCQ (0.158 μmol/L as compared to 1.17 μmol/L) (Figure 6B and D). No significant cytotoxic effects were observed when evaluated by the lactate dehydrogenase leakage (LDH) assays (Figure 2A and B). A 50% cytotoxic concentration (CC50 value) of 25 μmol/L was obtained for GNS-396 in the human liver slices culture at day 6 post-treatment. Similar CC50 values were obtained in proliferating Huh-7-5-1 replicon cells (23 μmol/L).

DISCUSSION

Our study evidenced that: (1) the *ex vivo* model of human liver slices HCVcc Con1 infection may be efficiently used for the assay of the antiviral potency of a new inhibitor (GNS-396 compared to HCQ) which interfered with autophagy; and (2) GNS-396 was a potent autophagy inhibitor, acting as "lysosomotropic agent" which was able to inhibit HCV replication in primary human adult HCVcc infected liver slices culture.

The establishment of the *ex vivo* model (feasibility, rapidity, specificity, potency) was already described in detail in 2012[41] with comparison between primary human hepatocytes, Huh-7.5.1 cell line. The Huh-7 cell system has several limitations that includes the inability to study the effects of pharmacologic inhibitors targeting the non-structural proteins of the most prevalent and problematic viral strains (*e.g.*, genotypes 1a and 1b). Moreover, the study of virus/host cell interactions is limited since the permissive cell lines are transformed and poorly differentiated. Firstly, the human liver slices culture maintains the original three-dimensional structure of the liver that allows cell crosstalk: The extra-cellular matrix and Kupffer cells essential for the normal function of the hepatocytes and the lobular structure. Secondly, the gene expression profiles in liver tissue slices were similar to that of the *in vivo* gene expression. Thirdly, primary hepatocytes preparations undergo treatment with collagenase (a treatment might have a negative effect on integrity of the proteins repertoire on the cell surface), but not the liver slices. Noteworthy, using established procedures, the tissue slices remained viable for, at least 10 d as it was shown by the secretion of albumin and urea. Moreover, the Huh-7 cell infection with primary isolates from patients are not very efficient. The infection of adult human liver slices culture allowed to achieve the robust replication of HCVcc genotype 2a, 1a and 1b genome and to obtain infectivity titers above 105 ffu/mL. In addition, we reported robust and productive infection using human primary isolates HCV genotype 1b. Stem cell-derived hepatocytes (hESC-Heps) displayed

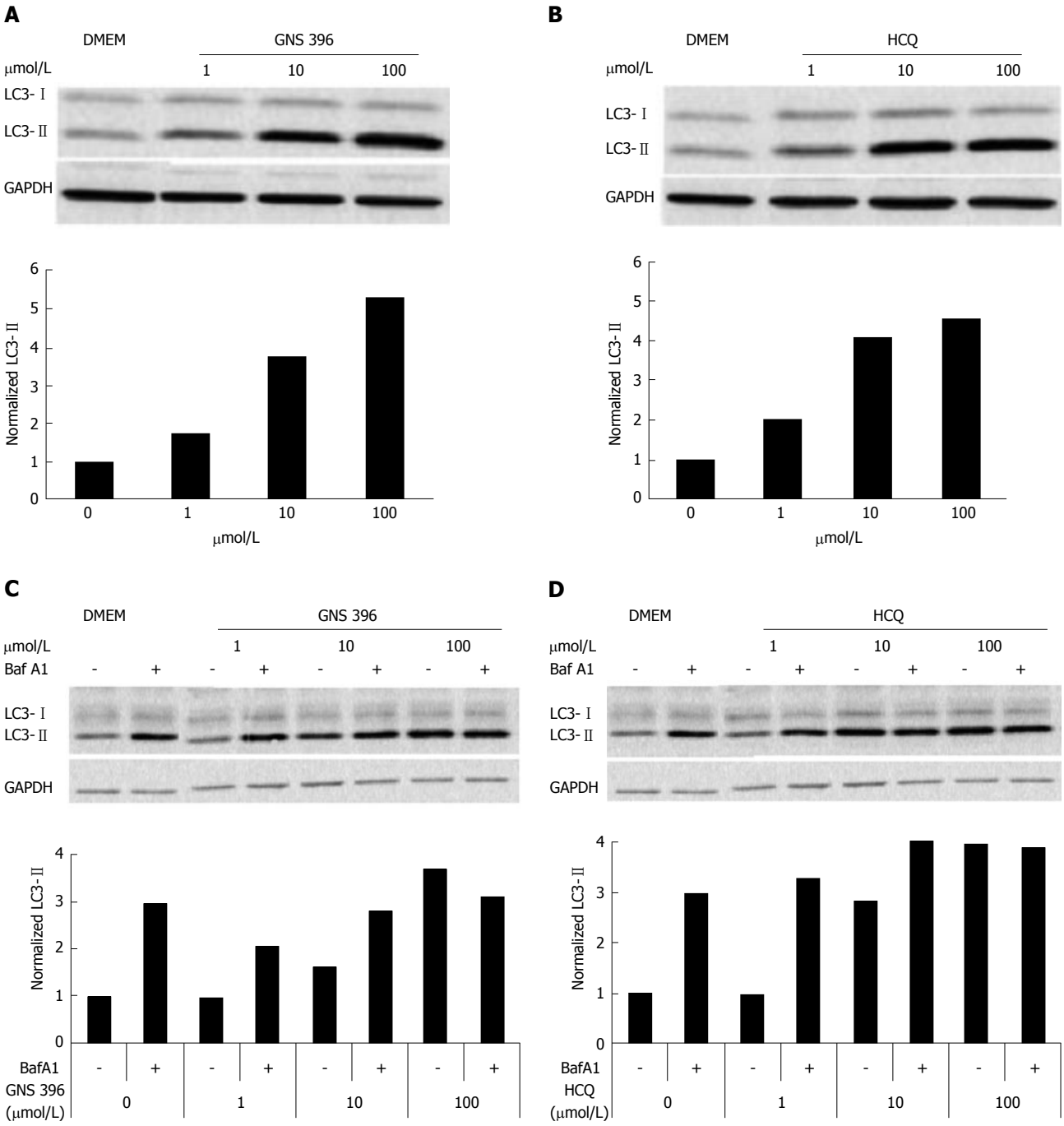

Figure 3 Modulation and inhibition of autophagy by treatment with GNS-396 in HeLa cells. A and B: Autophagy modulation was evaluated using HeLa cells treated either with (A) GNS-396 or (B) HCQ at different concentrations (1, 10 and 100 μmol/L) during 6 h. HCQ was used as a positive control of autophagy modulation. Intracellular expression of proteins LC3 was evaluated by Western-blot assay and normalized for LC3- Ⅱ; C and D: Autophagy inhibition by treatment with GNS-396 in HeLa cells. Autophagy inhibition was evaluated using HeLa cells treated either with (C) GNS-396 or (D) HCQ at different concentrations (1, 10 and 100 μmol/L) during 4 h in the presence or absence of 100 nmol/L bafilomycine A1. HCQ was used as a positive control of autophagy inhibition. LC3- Ⅱ intracellular expression was evaluated by Western-blot assay and normalized. HCQ: Hydroxychloroquine; LC3: Microtubule-associated protein 1A/1B-light chain; DMEM: Dulbecco's modified eagles's medium; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; BafA1: Bafilomycin A1.

equivalence to primary adult hepatocytes. HESC-Heps were capable of supporting the full HCV life cycle (JFH1), including the release of infectious virions. Although supportive, hESC-Hep viral infection levels were not as great as those observed in Huh7 cells. Up to now, the hESC-Heps were not infected with primary isolates[55]. Currently, we are establishing a culture of liver slices for 21 d, which allows us to follow the variation of different parameters and in particular, complete inhibition of viral production (data not shown).

Previous studies have reported that autophagy proteins are required to initiate HCV replication and translation[28,30-36]. Some data demonstrated that the suppression of LC3 protein lipidation, a necessary step for the formation of autophagosomes could also suppress HCV replication[30]. CQ is a well-known autophagic inhibitor

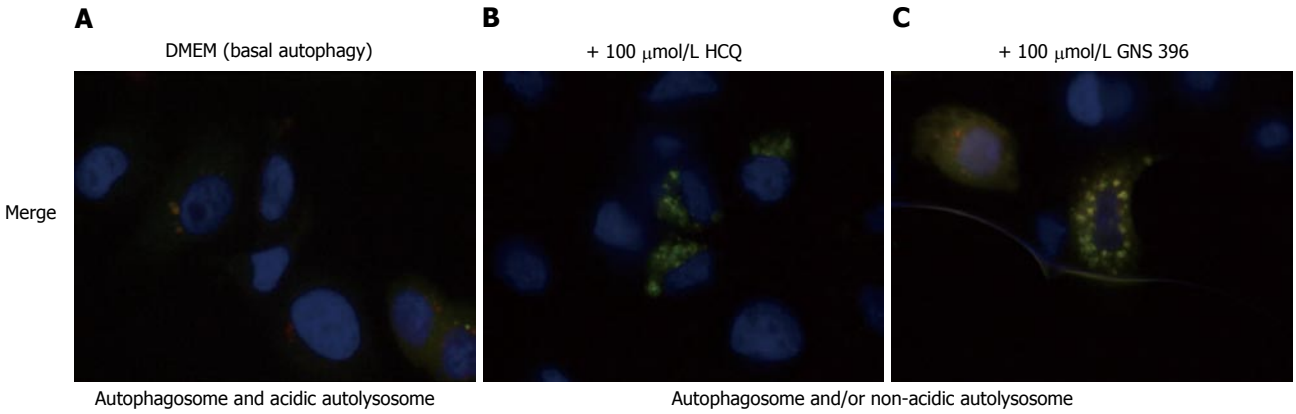

Figure 4 Inhibition of autophagic flux by treatment with GNS-396 in SkBr3 mRFP-EGFP-LC3 stable cell line. Autophagic flux was monitored using the mRFP-EGFP-LC3 tandem-tagged fluorescent protein in SkBr3 mRFP-EGFP-LC3 stable cell line. A: SkBr3 mRFP-EGFP-LC3 stable cell line without any treatment is representative of basal autophagy; SkBr3 mRFP1-EGFP-LC3 stable cell line was treated either with (B) 100 μmol/L HCQ or (C) 100 μmol/L GNS-396 during 6 h. In green/red merged images, yellow punctua (*i.e.*, mRFP+EGFP+) indicate autophagosomes or non-acidic autolysosomes, while red punctua (*i.e.*, mRFP+EGFP) indicate autolysosomes. HCQ is used as a positive control of autophagy inhibition. HCQ: Hydroxychloroquine; DMEM: Dulbecco's modified eagles's medium; LC3: Microtubule-associated protein 1A/1B-light chain.

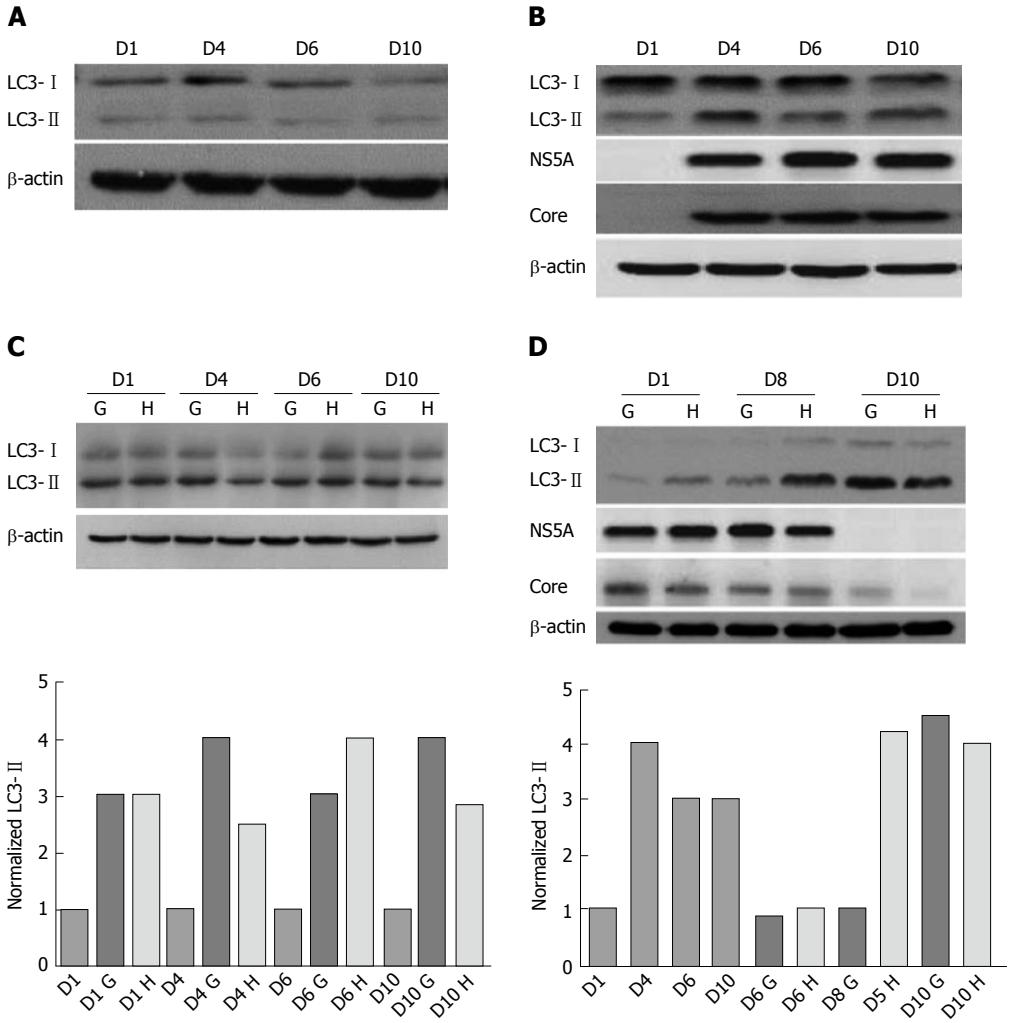

Figure 5 Inhibition of autophagy by treatment with GNS-396 (1 μmol/L) in primary adult human liver slices infected with cell culture-grown hepatitis C virus Con1 (multiplicity of infection = 1). A: Intracellular expression of LC3-Ⅰ/LC3-Ⅱ proteins in non-infected liver slices without treatment; B: Intracellular expression of LC3-Ⅰ/LC3-Ⅱ proteins and the normalization of intracellular protein LC3-Ⅱ expression, for 10 d in non-infected liver slices with treatment either by GNS-396 (1 μmol/L) (G) or HCQ (1 μmol/L) (H) or without treatment (D: day); C: Expression of LC3-Ⅰ/LC3-Ⅱ proteins and HCV core and NS5A proteins in HCVcc Con1 infected liver slices either without treatment or (D) either with treatment by GNS-396 (1 μmol/L) or HCQ (1 μmol/L) and the normalization of Intracellular protein LC3-Ⅱ expression for 10 d in HCVcc Con1 infected liver slices with treatment either by GNS-396 (1 μmol/L) (G) or HCQ (1 μmol/L) (H) or without treatment (D: day). LC3: Microtubule-associated protein 1A/1B-light chain; HCVcc: Cell culture-grown hepatitis C virus; NS5A: HCV nonstructural protein 5A.

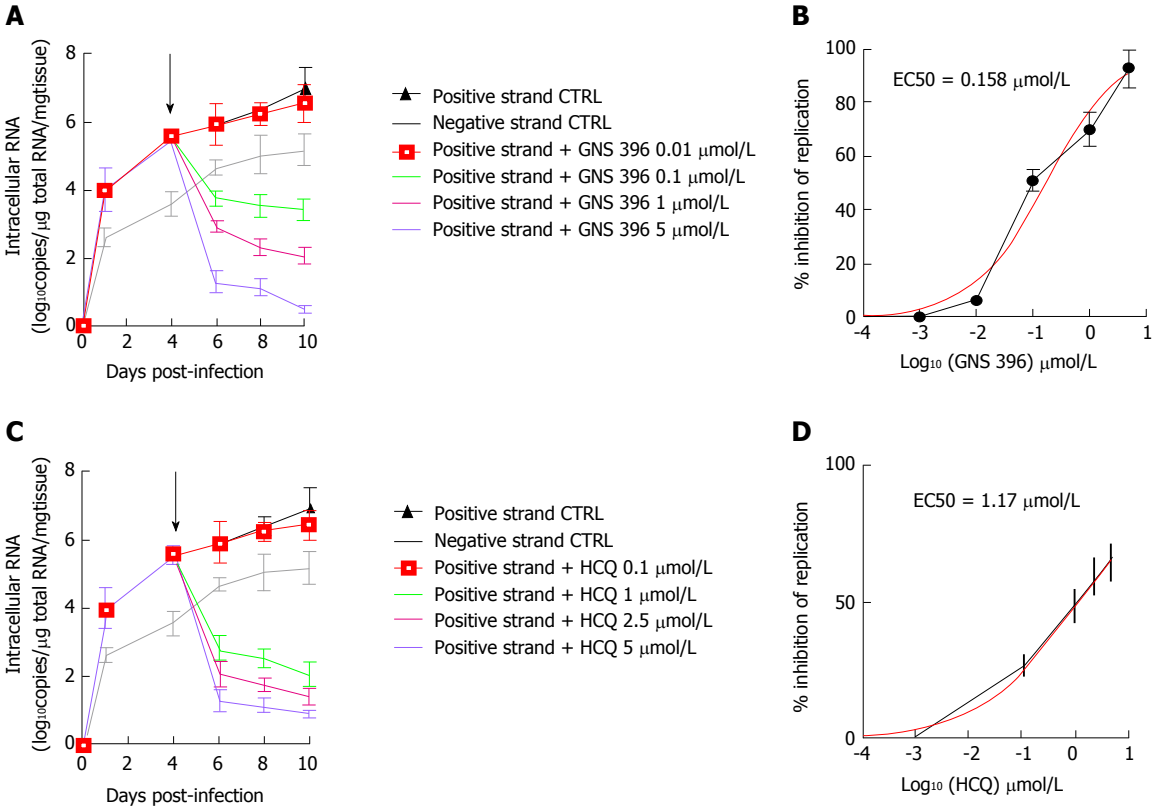

Figure 6 Inhibition of hepatitis C virus RNA replication by treatment either with GNS-396 or hydroxychloroquine in a dose-dependent manner in primary adult human cell culture-grown hepatitis C virus Con1 infected liver slices. Human liver slices were infected overnight with HCVcc Con1 (MOI = 0.1). The supernatant is then removed, the human liver slices washed and cultured. The liver slices and culture supernatants were collected different times post-infection. At day 4 post-infection, the liver slices were treated with increasing concentrations either of GNS-396 (0.01, 0.1, 1, 5 μmol/L) (A, B) or HCQ (C, D) for 6 d (black arrow: Start of the treatment either with GNS-396 or HCQ). Human HCVcc Con1 infected liver slices were lysed to evaluate intracellular levels of positive- and negative-strand HCV RNA by specific strand RT-qPCR at 1, 4, 6, 8, 10 d post-infection. The results were compared using the two-paired Student's test. Values are expressed as means ± standard errors: (A) HCV RNA replication by treatment with GNS-396: Positive strand (black line), $P < 0.03$; negative strand (grey line), $P < 0.013$, GNS-396 0.01 μmol/L (red line), $P < 0.04$; GNS-396 0.1 μmol/L (green line), $P < 0.05$; GNS-396 1 μmol/L (pink line), $P < 0.05$; GNS-396 5 μmol/L (blue line), $P < 0.05$; (C) HCV RNA replication by treatment with HCQ: Positive strand (black line), $P < 0.03$; negative strand (grey line), $P < 0.015$; HCQ 0.1 μmol/L (red line), $P < 0.0001$; HCQ 1 μmol/L (green line), $P < 0.0001$; HCQ 2.5 μmol/L (pink line), $P < 0.01$; HCQ 5 μmol/L (blue line), $P < 0.03$. The detection of negative strand of HCV RNA evidences active replication as well as the increase overtime of both positive and negative strands of HCV RNA; B: Inhibition of HCV replication (%) with GNS-396 treatment $P < 0.0038$; D: Inhibition of HCV replication (%) with HCQ treatment $P < 0.0013$. The replication was significantly inhibited in a dose-dependent manner in presence of increasing concentrations either of GNS-396 (B) or HCQ (D) for 6 d. HCVcc: Cell culture-grown hepatitis C virus; HCQ: Hydroxychloroquine; qRT-PCR: Quantitative technique consisting of reverse transcription followed by real-time polymerase chain reaction; MOI: Multiplicity of infection; CTRL: Control-treated liver slices.

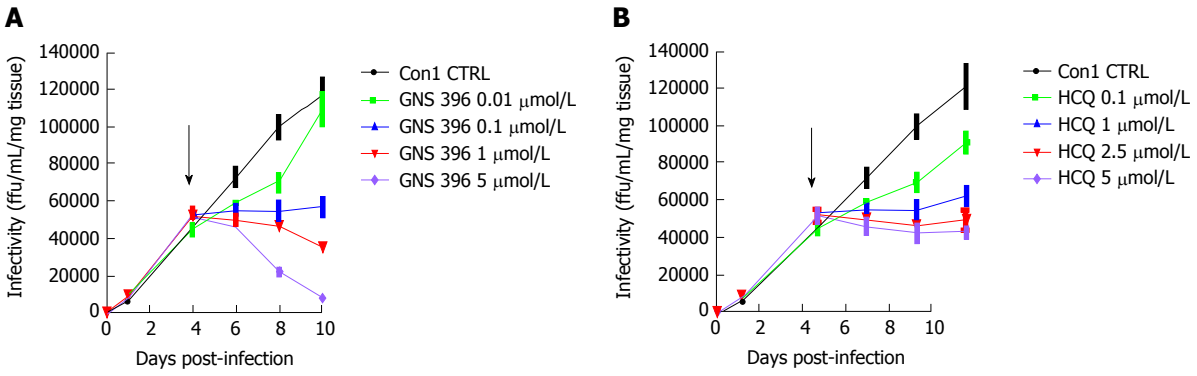

Figure 7 Dose-dependent inhibition of primary-culture-derived virus infectivity in primary adult human cell culture–grown hepatitis C virus Con1 infected liver slices by treatment either with GNS-396 (A) or hydroxychloroquine (B). Kinetics of infectivity of culture supernatants from human liver slices infected by HCV Con1 (MOI = 0.1) and treated either GNS-396 (A) or with HCQ (B) or at day 4 post-infection for 6 d. A: Con1 (black line), $P < 0.0001$; GNS-396 0.01 μmol/L (green line), $P < 0.0003$; GNS-396 0.1 μmol/L (blue line), $P < 0.019$; GNS-396 1 μmol/L (red line), $P < 0.05$; GNS-396 5 μmol/L (purple line), $P < 0.05$; B: Con1 (black line), $P < 0.0001$; HCQ 0.1 μmol/L (red line), $P < 0.0001$; HCQ 1 μmol/L (green line), $P < 0.0001$; HCQ 2.5 μmol/L (red line), $P < 0.0001$; HCQ 5 μmol/L (purple line), $P < 0.0003$. Each curve represented the average of 2 independent infections performed in triplicate from 2 different donors. Values are expressed as means ± SE. The results were compared using the two-paired student's test. HCV: Hepatitis C virus; HCQ: Hydroxychloroquine; MOI: Multiplicity of infection; CTRL: Control-treated liver slices.

Table 1 Inhibition of hepatitis C virus infectivity and 50% effective concentration of hepatitis C virus replication with direct active antivirals and autophagy inhibitors

Drugs[1]	Infectivity inhibition (%)	Average (SD)[2]	Replication inhibition (EC50)[3]	Average (SD)[4]	CC50 (SD)[5]
INF (2.6 to 260 nmol/L)[6]	Up to 95%	5	17 ng/mL	7.2	40 ng/mL (± 4)
RBV (1 to 100 μmol/L)	3% to 37%	3	146 μmol/L	13	400 μmol/L (± 21)
[7]TVR (0.01 to 50 μmol/L)	62% to 89%	4	0.395 μmol/L	0.038	40 μmol/L (± 3)
[7]BOC (0.01 to 50 μmol/L)	61% to 95%	5	0.417 μmol/L	0.024	41 μmol/L (± 5)
[7]SOF (0.01 to 50 μmol/L)	75% to 95%	4	0.147 μmol/L	0.017	23 μmol/L (± 2)
[7]HCQ (0.1 to 50 μmol/L)	25% to 94%	4	1.17 μmol/L	0.023	27 μmol/L (± 2)
[7]GNS-396 (0.01 to 5 μmol/L)	6% to 93%	3	0.158 μmol/L	0.014	25 μmol/L (± 2)
INF (2.6 to 260 nmol/L)/RBV 100 μmol/L	Up to 98%	6	10 ng/mL	3.1	43 ng/mL (± 4)
[7]HCQ (0.1 to 5 μmol/L)/RBV 50 μmol/L	27% to 85%	2	0.456 μmol/L	0.044	31 μmol/L (± 3)
[7]GNS-396 (0.01 to 5 μmol/L)/RBV 100 μmol/L	9% to 94%	2	0.157 μmol/L	0.012	26 μmol/L (± 2)
[7]TVR (0.01 to 50 μmol/L)/RBV 100 μmol/L	Up to 98%	3	0.310 μmol/L	0.029	49 μmol/L (± 3)
[7]BOC (0.01 to 50 μmol/L)/RBV 100 μmol/L	Up to 95%	2	0.370 μmol/L	0.035	48 μmol/L (± 4)
[7]SOF (0.01 to 50 μmol/L)/RBV 100 μmol/L	Up to 100%	2	0.080 μmol/L	0.028	17 μmol/L (± 5)
[7]TVR 1 μmol/L/7BOC 1 μmol/L	Up to 89%	2	0.410 μmol/L	0.039	50 μmol/L (± 3)
[7]TVR 1 μmol/L/INF 26 nmol/L/RBV 100 μmol/L	Up to 99%	3	0.315 μmol/L	0.031	44 μmol/L (± 4)
[7]BOC 1 μmol/L/INF 26 nmol/L/RBV 100 μmol/L	Up to 97%	2	0.350 μmol/L	0.033	47 μmol/L (± 3)
[7]SOF 1 μmol/L/INF 26 nmol/L/RBV 100 μmol/L	Up to 100%	3	0.055 μmol/L	0.029	18 μmol/L (± 3)

[1]Drugs added at day 4 post-infection for 6 d; [2]Average (SD) of infectivity inhibition at day 6 post-treatment; [3]EC50 of the drugs written in bold at day 6 post-treatment; [4]Average (SD) of EC50 at day 6 post-treatment; [5]CC50 (SD): 50% cytotoxic concentration of the drugs written in bold at day 6 post-treatment (standard deviation); [6]INF 26 nmol/L: Peg-INF concentration corresponding to SOC; [7]DAAs and autophagy inhibitors in bold. EC50: 50% effective concentration; BOC: An inhibitor of the HCV-encoded NS3 protein; TVR: An inhibitor of the HCV-encoded NS3/4A hepatitis C protease; SOF: An uridine analogue inhibitor of the HCV NS5B polymerase; HCV: Hepatitis C virus; TVR: Telaprevir; BOC: Boceprevir; SOF: Sofosbuvir; RBV: Ribavirin; SD: Standard deviation; Peg-INF: Pegylated-interferon α-2a; DAAs: Direct acting antivirals; HCQ: Hydroxychloroquine.

which is often used as an anti-malarial agent. HCQ is a "lysosomotropic" weak base that raises the lysosomal pH quickly[37]. Furthermore, many studies have reported the antiviral effect of CQ on other positive strand RNA viruses, such as polioviruses, coxsackieviruses, dengue viruses, coronaviruses (SARS-CoV virus)[24-29], HIV-1[56]. In our study, we demonstrated the antiviral effect of HCQ and the new quinoline derivative GNS-396 on HCVcc replication in a dose - dependent manner. Compared to the treatment with HCQ alone, HCQ inhibition was more pronounced in combination with RBV or with other direct antivirals, suggesting a synergistic effect of the combined drugs on HCVcc infection in human liver slices. This result is consistent with a previous study which demonstrated the antiviral effect of CQ in combination with peg-IFN in HCV infected Huh-7 cell line[33]. Similarly, on Huh-7 cells infected with HCVpp (genotype 1a and 3a), it has been shown that CQ reduced by 50% virus infectivity at 50 μmol/L concentration, when the antiviral effect was tested[57]. Recently, ferroquine (FQ), an antimalarial ferrocenic analog of CQ, has been described as a novel inhibitor of HCV. FQ potently inhibited HCV infection of hepatoma cell lines[58]. Compared to these investigations, our study using the quinoline derivative GNS-396, revealed an inhibition of the virus infectivity up to 93% respectively at day 6 post-treatment with lower drug amounts (EC50 = 0.158 μmol/L). This demonstrates that GNS-396 is a stronger antiviral than HCQ (EC50 = 1.17 μmol/L). EC50 is a measure of the effectiveness of the drug in inhibiting the biochemical function. In our study, we evaluated the EC50 of HCV replication at day 6 post-treatment. The lower EC50 value indicates the greater potency of inhibiting HCV replication. As shown in Table 1, the infectivity inhibition, consistent with the inhibition of HCV replication, demonstrated that the new drug evaluated in the human HCV infected liver slices culture model, had a potent antiviral effect compared to the well-known established antivirals. In combination with the other well established drugs like DAA or inhibitors of other host targets (cyclophilin), quinoline derivatives could be additional therapeutic options for HCV infected patients.

In conclusion, this study demonstrated the relevance of the human HCV infected liver slices culture in preclinical studies of the new anti-viral drugs. New host-targeted therapies inhibiting autophagy (GNS-396, HCQ) have demonstrated significant efficiency and additive activity in inhibiting HCV replication. The *ex vivo* model of culture of human HCV infected liver slices might allow further evaluation of the efficacy of new antiviral drugs in single or in combined therapy and their potential toxicity in particular for patients "difficult to treat". Moreover, the infection of human liver slices culture with primary viral isolates from patients that we succeed to establish[41], should allow highlighting the potential of early emergence of drug resistant viral variants during the anti-viral treatments.

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COMMENTS

Background

Hepatitis C virus (HCV) infection (or spread) is a serious public health challenge counting approximately 170 million people that are chronically infected worldwide. Host antiviral therapy is an additional option for the treatment of HCV infection.

Research frontiers

Interferon-free treatments with new direct acting antivirals are expected to cure more than 90% of HCV-infected patients. But they are not available in all the countries. At the present time, triple therapy combining pegylated interferon-α 2a or b and ribavirin with NS3 protease inhibitors (telaprevir or boceprevir) is going to remain the main treatment for HCV patients. That is why it appears important to continue research in limiting virus replication and the autophagy inhibition could be a new additional pathway because of recent evidences obtained regarding to an increased autophagic response in the liver of chronically HCV infected patients.

Innovations and breakthroughs

This is the first study evaluating a new autophagy inhibitor as antiviral that could inhibit HCV infection in a dose-dependent manner without cytotoxic effect using the relevant ex vivo model of the human liver slices culture.

Applications

This study highlight the relevance of the *ex vivo* model of the human HCV infected liver slices culture in preclinical studies of the new anti-viral drugs in single or in combined therapy and their potential toxicity in particular for patients "difficult to treat". Moreover, the infection of human liver slices culture with primary viral isolates from patients that the authors succeed to establish, should allow highlighting the potential of early emergence of drug resistant viral variants during the anti-viral treatments.

Terminology

Autophagy is a catabolic process which degrades a cellular own component through the lysosomal machinery. It has been shown that autophagy is activated during virus and bacterial infection and that some viruses can use the autophagy system to facilitate their own replication.

Peer-review

The manuscript is clear and comprehensive.

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