Amiodarone inhibits the entry and assembly steps of hepatitis C virus life cycle

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Abstract
HCV (hepatitis C virus) infection affects an estimated 180 million people in the world’s population. Adverse effects occur frequently with current standard treatment of interferon and ribavirin, while resistance of new direct anti-viral agents, NS3 protease inhibitors, is a major concern because of their single anti-HCV mechanism against the viral factor. New anti-viral agents are needed to resolve the problems. Amiodarone, an anti-arrhythmic drug, has recently been shown to inhibit HCV infection in vitro. The detailed mechanism has yet to be clarified. The aim of the present study was to elucidate the molecular mechanism of the inhibitory effect of amiodarone on HCV life cycle. The effect of amiodarone on HCV life cycle was investigated in Huh-7.5.1 cells with HCVcc (cell culture-derived HCV), HCVpp (HCV pseudoviral particles), sub-genomic replicons, IRES (internal ribosomal entry site)-mediated translation assay, and intracellular and extracellular infectivity assays. The administration of amiodarone appeared to inhibit HCV entry independent of genotypes, which was attributed to the down-regulation of CD81 receptor expression. The inhibitory effect of amiodarone also manifested in the HCV assembly step, via the suppression of MTP (microsomal triacylglycerol transfer protein) activity. Amiodarone revealed no effects on HCV replication and translation. With the host factor-targeting characteristics, amiodarone may be an attractive agent for the treatment of HCV infection.

Key words: amiodarone, CD81, entry and assembly, hepatitis C virus (HCV), life cycle, microsomal triacylglycerol transfer protein (MTP)

INTRODUCTION
HCV (hepatitis C virus) was identified in 1989 [1] and has since been identified as a major cause of chronic hepatitis [2]. The positive-stranded RNA genome of HCV can be translated into at least ten proteins. Structural proteins include core, E1 (envelope) and E2, whereas non-structural proteins are p7, NS2 (non-structural protein 2), NS3, NS4A, NS4B, NS5A and NS5B [3]. Six genotypes of HCV and several sub-genotypes have been identified worldwide [4].

The standard treatment for chronic HCV infection is the combination of pegylated-interferon with ribavirin, which is only effective in 50% of patients and is associated with significant side effects [5]. The newly developed DAA (direct-acting anti-virals), boceprevir and telaprevir, known as NS3 protease inhibitors, have been approved by the FDA (Food and Drug Administration) for genotype 1 chronic hepatitis C [6]. However, the high tablet load and patient compliance are noteworthy problems. The development of resistance is also an important issue. Accordingly, it is imperative to develop novel anti-HCV agents based on targeting different steps of the HCV life cycle that have better efficacy and fewer side effects.

In the past two decades, understanding of the HCV life cycle has progressed significantly because of the establishment of sub-genomic replicon systems [7], HCVpp (HCV pseudoviral particles) [8] and HCVcc (cell culture-derived HCV) systems [9–11]. The HCV life cycle starts from the binding of HCV particles with host cell receptors, such as CD81, LDL
(low-density lipoprotein) receptor, occludin, claudin-1 and SR-B1 (scavenger receptor class B type 1). Then, translation and replication occur in the cytoplasm and endoplasmic reticulum, followed by assembly and release, which is related with the VLDL (very-low-density lipoprotein) biosynthesis [3]. VLDL assembly and secretion depend on the interaction of apolipoproteins and MTP (microsomal triglyceride transfer protein) [12]. Reduced infectious virus titres from cells treated with an MTP inhibitor without any effect on viral RNA replication suggests that MTP activity is required for HCV assembly as well as secretion [13].

Amiodarone belongs to the family of CAD (cationic amphiphatic drugs) and interacts preferentially with lipid membranes to exert its anti-arrhythmic effect [14]. The ability of amiodarone to suppress HCV infection has recently been reported [15,16]. Amiodarone inhibited MTP activity in vitro, and decreased ex vivo MTP activity in the hepatic homogenate of treated mice [17], which may account for its inhibitory effect on HCV infectious virus production. However, the direct link between down-regulation of MTP activity by amiodarone and reduction of HCV infectious virus production has not been proved.

The present study aimed to analyse which steps of HCV life cycle were targeted by amiodarone and the related molecular mechanism. The result demonstrated that amiodarone could reduce HCV infectivity with limited cytotoxicity by inhibiting HCV entry via decreasing the expression of CD81 receptor, and assembly and release via decreasing the MTP levels.

MATERIALS AND METHODS

In vitro transcription and infectious HCV production

HCV Con1 replicons (pFK-I389-luc-NS3-3/5.1, pFK-I389-luc-ubi-neo/NS3-3/5.1) [18] and JFH1 constructs (pSGR-Luc-JFH1 [19], J6/JFH and J6/JFH (p7-Rluc2A) [20]) were digested with ScaI and XbaI, respectively, for linearization. In vitro transcription and infectious HCV production were performed as described previously [21].

Cell cultures and reagents

Human hepatoma cell line Huh-7.5.1 [22] and HEK (human embryonic kidney)-293T cells were grown in DMEM (Dulbecco’s modified minimal essential medium; Life Technologies) supplemented with 2 mM l-glutamine, non-essential amino acids, 100 unit/ml penicillin, 100 mg/ml streptomycin and 10% (v/v) FBS (fetal bovine serum). Huh-7.5.1/pFK-I389 luc-ubi-neo/NS3-3/5.1 stable cells were established by transfection of pFK-I389 luc-ubi-neo/NS3-3/5.1 RNA into Huh-7.5.1 cells following selection with G418 (500 μg/ml) for 3 weeks. Amiodarone was obtained from Wyeth-Ayerst Laboratories.

Drug inhibition study

Amiodarone was resuspended at 10 mM in DMSO. Huh-7.5.1 cells were seeded in a 96-well plate at a density of 10^4 cells per well. After 24 h, Huh-7.5.1 cells were pre-treated for 1 h with 50 μl of 2.4, 7.4, 22 and 66 μM amiodarone or an equivalent volume of DMSO, diluted in the complete growth medium. The cells were then incubated with an equal volume of J6/JFH (p7-Rluc2A), bringing the final concentration of amiodarone to 1.2, 3.7, 11 and 33 μM. The infection was allowed to proceed in the presence of the compounds for 48 h before measuring luc (luciferase) activity.

Huh-7.5.1/pFK-I389 luc-ubi-neo/NS3-3/5.1 cells seeded at a density of 1×10^5 cells/well on a 12-well plate were incubated with indicated concentrations of amiodarone (3 and 6 μM) or DMSO control. After 48 h, the cells were subjected to the luc assay.

Cell viability assay

Huh-7.5.1 cells seeded in the 96-well plate were treated with the DMSO control or indicated concentrations of amiodarone followed by infection with J6/JFH (p7-Rluc2A). After 48 h, the cell viability assay was performed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay as described previously [21].

HCV reporter pseudovirus production and amiodarone treatment

Pseudo-typed viruses were produced following the protocols reported previously [21]. Huh-7.5.1 cells seeded in a 96-well plate were pre-treated for 1 h with medium containing amiodarone (6 and 12 μM) or DMSO (0.2%). The cells were infected with an equal volume of different pseudo-particles. Firefly luc assay was performed 48 h later.

Transient transfection and luc assay

Huh-7.5.1 cells seeded at a density of 10^6 cells in a 10-cm dish were transfected with 10 μg of pRL-CMV-1-515 [23], in which the firefly luc gene was driven by the HCV IRES (internal ribosome entry site) nt 1–515 using FuGene6 (Roche) following the manufacturer’s instruction. After 24 h, the transfected Huh-7.5.1 cells were trypsinized and plated into a 96-well plate at a density of 10^3 cells per well and incubated with complete growth medium containing 0.1% DMSO with or without amiodarone (3 and 6 μM) for 48 h. The cells were subjected to luc assay as described above.

Western blot analysis

Huh-7.5.1 cells were treated with DMSO (0.1%) or the indicated concentrations of amiodarone for 48 h. The cells were harvested and 100 μg of each sample was subjected to immunoblotting using antibodies against occludin (anti-mouse, clone OC-3F10; Zymed; Invitrogen), SR-B1 (anti-rabbit; epitomics) or LDL receptor-specific antibodies (anti-rabbit, epitomics). The blots were reprobed with β-actin-specific antibodies (mouse anti-β-actin; Sigma).

Huh-7.5.1 cells were infected with J6/JFH viruses and then incubated with the medium containing the indicated concentrations of amiodarone for 48 h. The cells were harvested and 50 μg of each sample was subjected to immunoblotting using antibodies directed against proteins of interest: HCV NS3 (mouse anti-HCV NS3 clone 8G2; Abcam), HCV NS5A (mouse anti-HCV NS5A clone 9E10), HCV NS5B (rabbit anti- HCV NS5B; abcam) and β-actin (mouse anti-β-actin; Sigma). The relative band intensity was quantified by densitometric analysis with ImageJ software (National Institutes of Health).
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Flow cytometry
Huh-7.5.1 cells were treated with DMSO (0.1%) or indicated concentrations of amiodarone for 24 or 48 h followed by staining with FITC-conjugated mouse anti-human CD81 (clone JS-81; Becton Dickinson) or its isotope control (Mouse IgG1, κ Isotype; Becton Dickinson) antibodies. The surface immunofluorescence was measured with an FACS-Calibur flow cytometer (Becton Dickinson) as described [21]. Three independent experiments were performed. Huh-7.5.1 cells infected with lentivirus bearing shRNA (small-hairpin RNA) against CD81 (National RNAi Core Facility, Academia Sinica, Taiwan) followed by puromycin (2 μg/ml) selection were stained with FITC-conjugated mouse anti-human CD81 or its isotope control IgG and the surface immunofluorescence was measured as described above.

Intracellular and extracellular infectivity
Huh-7.5.1 cells plated on 12-well plate were infected with J6/JFH overnight and the medium was replaced with DMSO or indicated concentrations of amiodarone. Culture media were then changed to fresh DMEM/10% (v/v) FBS the following day. After incubation for another 24 h, culture media were collected followed by centrifugation. The resulting supernatant was used as the extracellular HCV source. Cell-associated infectivity tests were performed as described, with modification [24, 25]. Briefly, the cells were washed twice in PBS and trypsinized. The cells were then collected by centrifugation at 5000 g for 5 min and resuspended in 1 ml of DMEM/10% (v/v) FBS and lysed by four cycles of freezing and thawing in liquid nitrogen and 37°C water bath. The lysate supernatant was collected after centrifugation, and was used as the intracellular HCV source. Intracellular and extracellular released virions were used to infect naïve Huh-7.5.1 cells plated on a 12-well plate. After an additional 48 h incubation, the cells were stained with anti-NS3 (clone 8G2; Abcam) followed by staining with an Alexa Fluor® 488 goat anti-mouse secondary antibodies (A-11001, Invitrogen) and staining for DNA with DAPI (4′,6-diamidino-2-phenylindole). The cells were then imaged on a fluorescence microscope (Zeiss Axio Observer A1), using two wavelengths, 350 nm for nuclear DNA bound by DAPI and 488 nm to detect infected cells expressing HCV NS3, to determine the total cells per well and the HCV-infected cells in each well, respectively. The relative intracellular and extracellular infectivity were determined by NS3-expressing cell numbers divided by total cell numbers (positive DAPI-stained cells) using Image J software.

Huh-7.5.1 cells plated on 12-well plate were infected with lentivirus-carrying shRNA against the control luc or MTTP gene (National RNAi Core Facility, Academia Sinica, Taiwan) at a multiplicity of infection of 6 followed by selection with puromycin (2 μg/ml) for 72 h. The cells were infected with J6/JFH virus in the absence or presence of amiodarone followed by the collection of intracellular virions by freeze and thaw treatment 48 h later. The intracellular HCV particles were used to inoculate naïve Huh-7.5.1 cells for 48 h. The relative intracellular infectivity was determined as described above.

MTP protein activity assay
MTP protein activity was measured using a commercial kit (Roar Biomedical). Huh-7.5.1 cells treated with the DMSO control or amiodarone at the indicated concentrations were scraped into PBS on ice, centrifuged at 600 g for 3 min and resuspended in buffer supplemented with protease inhibitor cocktail (Roche). The cell suspensions were sonicated on ice. Cell lysates (100 μg) were combined with 10 μl of donor and acceptor particles in 220 μl of assay buffer, and incubated at 37°C. The increase in fluorescence was measured using a FLUOstar Optima microplate reader with excitation and emission of 465/538 nm.

Human ApoE (apolipoprotein E) ELISA
Following the treatment with DMSO control or the indicated concentrations of amiodarone, Huh-7.5.1-secreted ApoE was detected in the medium using an ApoE human ELISA kit (Abcam). The medium was diluted 1:10 with the specimen diluent and the assay was carried out according to the manufacturer's directions.

Statistical analysis
Data analysis was performed using the GraphPad Prism 5. A post-hoc Student’s t test was used for comparisons. A P < 0.05 was considered statistically significant.

RESULTS
HCV infectivity was inhibited by amiodarone
J6/JFH (p7-Rluc2A) reporter virus was used to evaluate the effect of amiodarone on HCV infectivity. Relative to DMSO-treated controls, amiodarone treatment at non-cytotoxic concentrations inhibited HCV infectivity in a dose-dependent manner (Figure 1A), with high potency [the EC50 (half maximal effective concentration) was 2.1 μM and the 90% EC90 (effective concentration) 8.6 μM] and low cytotoxicity [the LD50 (median lethal dose) 44.4 μM]. The therapeutic index (LD50/EC50) was 20.6 (Figure 1B).

HCV entry was inhibited by amiodarone
HCV pseudo-particles of different genotypes were used to analyse the effects of amiodarone on HCV entry. After the treatment with amiodarone, HCVpp infectivity decreased to between 44.3 and 53.3% at 3 μM and 35.7 and 44.7% at 6 μM (Figure 2A), depending on different genotypes. The entry of VSVpp (vesicle stomatitis virus pseudo-typed virus) was not changed by amiodarone treatment, suggesting the inhibition of HCV entry by amiodarone as HCV-specific. The expression level of cell receptors essential for HCV entry following amiodarone treatment was also investigated in Huh-7.5.1 cells. Although SR-B1, claudin-1 and occludin receptor expressions increased after amiodarone treatment (Figures 2B–2D), the CD81 expression was down-regulated in a dose-dependent manner, as determined by flow cytometry (Figure 3A). To better characterize the weight of CD81 reduction attributed to amiodarone, we further investigated the time course of CD81 inhibition by amiodarone. While only mild reduction of CD81 expression occurred 24 h after
amiodarone treatment, the expression of CD81 was significantly inhibited 48 h after treatment from 66.2 % (without amiodarone) to 48.2 and 34.3 % (P = 0.0012) at 3 and 6 μM, respectively (Figure 3B). To demonstrate the direct mechanistic link of HCVpp entry inhibition and CD81 reduction induced by amiodarone, we established a CD81-negative Huh-7.5.1 cells by lentivirus carrying shCD81 following puromycin selection. The CD81 knockout effect was verified by flow cytometry (Figure 3C). The HCVpp infectivity in CD81-negative Huh-7.5.1 cells was low and independent of amiodarone concentrations (Figure 3D), suggesting that reducing CD81 expression limits the effects of amiodarone on HCV entry, thus ruling out other possible mechanisms.

HCV replication and IRES-mediated translation were not inhibited by amiodarone

Huh-7.5.1 cells stably harbouring HCV sub-genomic Con1 replicons, pFK-I389luc-NS3-3'5.1, were used to evaluate the effects of amiodarone on HCV replication. Following amiodarone administration, the luc activity of HCV Con1 replicon (Figure 4A) did not reveal significant changes compared with the DMSO control. To investigate the effect of amiodarone on HCV IRES-mediated translation, Huh-7.5.1 cells were transfected with pRL-CMV-1-515, in which firefly luc activity was governed by the HCV-IRES nt 1–515. The HCV-IRES-mediated translation activity was 118 and 87 % in cells treated with amiodarone at 3 and 6 μM, respectively, compared with those treated with the DMSO control (Figure 4B).

The NS3 and NS5B expressions were measured in Huh-7.5.1/pSGR-JFH1 cells after amiodarone treatment (Figure 4C). Amiodarone had no effects on the expression of non-structural proteins. Taken together, amiodarone had no effect on HCV replication and translation.

HCV assembly and release was inhibited by amiodarone

IC (intracellular) and EC (extracellular) virus particles have been proven to be infectious in vitro and the infectivity could be measured [26]. Both IC and EC infectivity were inhibited by amiodarone in a dose-dependent manner, with 33 % (P = 0.03) and 54 % (P = 0.001) reduction in IC and 16 % (P = 0.004) and 35 % (P = 0.008) reduction in EC infectivity by amiodarone at a concentration of 3 and 6 μM respectively (Figure 5A). These results provided evidence that amiodarone inhibited both assembly and release.

Since VLDL formation was associated with HCV assembly and release, and MTP and ApoE were of importance to the process [12,27–29], commercial kits were used to quantify their levels. MTP activity was reduced to 69.9 and 52 % in 3 and 6 μM amiodarone respectively (Figure 5B). However, ApoE had an insignificant increasing trend (Figure 5C). These findings suggested that amiodarone suppressed MTP activity to down-regulate HCV assembly and release. To elucidate the link between the effect of amiodarone and MTP expression on HCV assembly, we used irrelevant luc shRNA as a control and MTP shRNA to silence MTP expression, respectively, in Huh-7.5.1 cells followed by infection with J6/JFH virus and treatment with increasing concentration of amiodarone. Although amiodarone dose-dependently inhibited intracellular HCV assembly in Huh-7.5.1 cells infected with lentivirus-bearing irrelevant luc shRNA, the intracellular HCV assembly was not affected by amiodarone in cells silenced with MTP shRNA (Figure 5D). The result provided a mechanistic link between amiodarone and MTP expression on HCV assembly.

DISCUSSION

HCV infection affects about 3 % of the world’s population [30]. Current standard treatment with interferon and ribavirin has
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Serious adverse effects, while resistance of new direct anti-viral agents, NS3 protease inhibitors, is a major concern due to their single anti-HCV mechanism against the viral factor [6]. New anti-viral agents are needed to resolve the problems. Two recent cell-based screenings for anti-HCV agents have identified that amiodarone can inhibit HCV reproduction [15,16]. However, the detailed mechanism involved has not been well established. Consistent with a previous report, the present study shows that amiodarone inhibits HCV production and entry but has no effect on HCV replication [16]. Most importantly, amiodarone suppresses HCV entry and assembly steps via suppressing CD81 receptor expression and MTP activity, respectively.

The entry of HCV into target cells starts from binding with host cell receptors, such as CD81, occludin, claudin-1, LDL receptor, and SR-B1, followed by the fusion of viral and endosomal membranes. By utilizing the HCV pseudo-typed virus particle, amiodarone is shown to inhibit the entry of HCVpp, but not VSVpp, suggesting that this inhibitory effect is HCV-specific. Furthermore, the entry of all sub-genotypes of HCVpp tested (i.e., genotypes 1a, 1b and 2a) is profoundly inhibited by amiodarone at a concentration of 5 μM.

To determine the mechanism of the inhibitory effect of amiodarone on HCV entry, the expression level of several HCV entry factors like SR-B1, claudin-1, and occludin after amiodarone treatment was investigated. At concentrations of 3 and 6 μM, amiodarone increased SR-B1, claudin-1 and, to a lesser extent, occludin, expression, as demonstrated by immunoblotting (Figure 2B). In contrast, CD81 receptor expression was downregulated after amiodarone administration in a dose-dependent manner, as shown by flow cytometry (Figure 2C). Since CD81 is the most important receptor for HCV entry, its suppression by amiodarone may be responsible for the inhibitory effect of amiodarone on the HCV entry step [31].

Several compounds have been shown to inhibit HCV infection via suppression or interaction with CD81 receptor. Interferon-α, the crucial component of current standard treatment for HCV infection, has revealed its inhibitory effect on CD81 expression in vitro and in vivo [32]. Seven compounds synthesized from imidazole, an anti-fungal agent with CD81-mimic structure, have been proven to possess the ability to reversibly bind to HCV E2 and disrupt the attachment of HCV to host cells [33]. With the same mechanism, terfenadine, an anti-histamine, and salicylate can inhibit HCV infection via disruption of the binding between HCV E2 and CD81 [34,35]. The results here may add amiodarone as a new member that inhibits HCV entry via modulation or/and interaction with the CD81 receptor. Aside from decreasing the
expression of CD81 receptor, amiodarone also reduces mRNA and protein level of LDL receptor in vivo [36,37]. Thus the down-regulation of both CD81 and LDL receptors may be responsible for the inhibitory effect of amiodarone on the HCV entry step.

Chockalingam et al. [16] used the chimaeric genotype 2a HCV, Jc1, to assess the influence of amiodarone on HCV replication and observed that HCV replication was not affected. Utilizing sub-genomic HCV 1b replicons derived from Con1 strain, replication was shown to be undisturbed by amiodarone treatment, which further validates the observation that amiodarone has no effect on HCV replication regardless of genotypes. Similarly, IRES-mediated translation is not affected as demonstrated by reporter assay using a construct with luc gene driven by HCV-IRES sequences and immunoblotting for non-structure protein expression.

The present study demonstrates that both intracellular and extracellular infectivity of HCV is significantly suppressed by amiodarone, indicating that amiodarone inhibits HCV assembly, and consequently, its downstream release step. To elucidate the molecular mechanism of the inhibitory effect of amiodarone on HCV assembly and egress, the effects of amiodarone on MTP activity and ApoE production have also been investigated. Both are main components involved in the late stage of the HCV life cycle [29]. At a concentration of 6 μM, amiodarone decreased MTP activity to 52%, consistent with previous research showing that amiodarone reduces MTP activity in mice [17].

In contrast, amiodarone treatment augmented ApoE production at concentrations of 3 and 6 μM, but not statistically significantly. These results suggest that amiodarone suppresses only MTP activity, but not ApoE production, to inhibit HCV assembly and its downstream egress step. Whereas toxic fat accumulation in long-term use is a concern, MTP inhibitors have the potential to treat HCV infection with short-term use [38]. Amiodarone, a potential MTP inhibitor, may be enlisted in the category for further study.

Medications targeting host factors, such as CD81 and MTP as amiodarone does, may be an ideal prototype for the development of anti-HCV agents as long as they have few side effects. Gastaminza et al. [15] reveal that amiodarone has limited cytotoxicity (95.7% cell biomass) at 10 μM and can inhibit infectious viral
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Figure 4 Impact of amiodarone on HCV translation and replication

(A) Huh-7.5.1/pFK-I389 luc-ubi-neo/NS3-3/5.1 cells were incubated with 0.2% DMSO or indicated concentration of amiodarone for 48 h. luc activity was measured as representative of HCV replication efficiency against DMSO control. (B) Huh-7.5.1 cells were transfected with pRL-CMV-1-515 and then incubated with medium containing DMSO or indicated concentrations of amiodarone. luc activity was measured as representative of HCV translation efficiency against the DMSO control. (C) Huh-7.5.1 cells stably transfected with pSGR-JFH were treated with indicated concentrations of amiodarone for 48 h. Following lysis buffer treatment, 50 μg of cell lysates were subjected to immunoblotting using anti-NS3 (top panel) and anti-NS5B antibodies (middle panel), and reprobed with anti-β-actin antibodies (bottom panel).

production by over 80% at 5 μM with a therapeutic index up to 23.3 [15]. A similar study result shows that the cell viability is more than 80% at a concentration of 10 μM amiodarone with undetermined therapeutic index [16]. Our study had a comparable result showing no obvious cytotoxicity by amiodarone at a concentration of 11 μM, with a therapeutic index of 20.6. The recommended therapeutic range of amiodarone in serum is between 1.5 and 4 μM without leading to dose-dependent adverse effects [39]. Amiodarone serum >4.0 μM has been reported as being associated with the side effects such as deterioration of vision and polyserositis; however, as pointed out by the authors, most patients had complex co-morbidities and the data may not be representative for otherwise healthy subjects [39]. Furthermore, the incidence of amiodarone-related adverse effects was significantly associated with the cumulated time of treatment and the cumulated dose received, with more than 2 years or 145 g of cumulated dose on amiodarone [40]. At a concentration of 3 μM amiodarone, within the recommended therapeutic range of serum amiodarone concentrations between 1.5 and 4 μM, our results revealed statistically significant inhibition of HCVpp entry (Figure 2A) and IC and EC HCVcc infectivity (Figure 5A). Based on limited adverse effects for short-term use and good efficacy on inhibiting HCVcc, with EC50 equal to 2.1 μM in our study, amiodarone may be a potential and attractive agent for treating HCV infection.

In conclusion, amiodarone has inhibitory effects on HCV infection through at least two steps of the HCV life cycle, entry and assembly, by down-regulating CD81 receptor expression and MTP activity, respectively. Since CD81 receptor and MTP are both essential host factors involved in the HCV life cycle, drug resistance may not be an issue if a compound like amiodarone, targeting CD81 or/and MTP, is applied for anti-HCV treatment. With the host factor-targeting characteristics, amiodarone may offer an advantage in treating HCV infection. Further studies in animal models and clinical trials are needed to extend the current results into clinical application.

CLINICAL PERSPECTIVES

- Current standard treatment with interferon and ribavirin has serious adverse effects, while resistance of new direct
Figure 5 Impact of amiodarone on HCV assembly and release

(A) Huh-7.5.1 cells were infected with J6/JFH virus one day before inoculation with medium containing 0.2% DMSO and indicated concentrations of amiodarone. After 48 h, the supernatant was collected for use as extracellular HCV source. Cell-associated HCV particles were collected from cell lysates following treatment with four freeze-thaw cycles. Following infection of naïve Huh-7.5.1 cells with the collected intracellular and extracellular virions, relative intracellular and extracellular infectivity was determined by NS3-expressing cell numbers divided by total cell numbers (positive DAPI-stained cells) using Image J software. (B) Huh-7.5.1 cells were treated with amiodarone or DMSO control for 24 h. The MTP activity was measured using a commercial MTP activity assay kit. (C) Huh-7.5.1 cells were treated with DMSO control or indicated concentrations of amiodarone for 48 h. Huh-7.5.1-secreted ApoE concentrations were determined using a commercial kit. (D) Reduction of MTP expression blunts the impact of amiodarone on intracellular HCV infectivity. Huh-7.5.1 cells infected with lentivirus carrying control luc or MTP shRNA followed by selection with puromycin for 72 h were infected with J6/JFH virus and treated with amiodarone at the indicated concentrations for additional 48 h. Intracellular HCV particles were collected from cell lysates following four cycles of freeze and thaw treatment. Following infection of naïve Huh-7.5.1 cells with the collected intracellular virions, relative intracellular infectivity was determined by NS3-expressing cell numbers divided by total cell numbers (positive DAPI-stained cells) using Image J software. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared with control, by Student’s t test.

anti-viral agents is a major concern due to their single anti-HCV mechanism against the viral factor. Two recent cell-based screenings for anti-HCV agents have identified that amiodarone can inhibit HCV reproduction.

- The aim of the present study was to dissect the detailed mechanism of amiodarone inhibition of HCV infection with systems representing different steps of HCV life cycle, and analyse the molecular aspect responsible for the suppression of each inhibited step. Amiodarone targets entry and assembly steps of the HCV life cycle to inhibit HCV infection by down-regulating CD81 receptor expression and suppressing MTP activity respectively.

- On the basis of its host factor-targeting effect on HCV infection, amiodarone may serve as a candidate to combine with conventional anti-HCV therapy to potentiate therapeutic efficacy.

AUTHOR CONTRIBUTION

Keng-Hsin Lan was involved in the study concept and design, critical revision of the paper for important intellectual content, obtaining funding for the study and study supervision; Yuan-Lung Cheng was involved in the manuscript drafting; Keng-Hsueh Lan and Wei-Ping Lee were involved in data analysis and interpretation. Szu-Han Tseng and Li-Rong Hung offered technical support. Han-Chieh Lin, Fa-Yauh Lee, and Shou-Dong Lee were involved in study supervision.

ACKNOWLEDGEMENTS

We thank all of those who provided essential materials used in this study: Dr Francis V. Chisari (The Scripps Research Institute, La Jolla, CA, U.S.A.) for the human hepatoma cell line Huh-7.5.1; Dr Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany) for the J6/JFH virus.
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Germany) for the pFK-1389Luc-NS3-3/5.1 and pFK-1389Luc-ubino/NS3-3/5.1; Dr John MaLauchlan (MRC Virology Unit, Institute of Virology, Glasgow, U.K.) for the pSGR-Luc-JFH1; Dr Charles M. Rice (The Rockefeller University, New York, NY, U.S.A.) for both J6/JFH and J6/JFH(p7-Rluc2A) plasmids and anti-HCV NSSA antibody clone 9E10; Dr Susan L. Uprichard (University of Illinois at Chicago, Chicago, IL, U.S.A.) for the JFH-1 E1/E2 glycoprotein expression plasmid (pCDNA3.1_JFHcE1/E2); Dr T. Jake Liang (LDB, NIDDK, NIH, Bethesda, MD, U.S.A.) for the plasmids encoding E1/E2 glycoproteins from HCV genotype 1a or 1b; FL Cosset (INSERM U412, Lyon, France) for the expression plasmid encoding the vesicular stomatitis virus glycoprotein.

FUNDING
This study was supported by the National Science Council (NSC) [grant number 101-2314-B-075 -014] and Taipei Veterans General Hospital, Taipei, Taiwan [grant number V101C-112].

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Received 1 November 2012/4 April 2013; accepted 9 May 2013
Published as Immediate Publication 9 May 2013, doi: 10.1042/CS20120594