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註：研究代表者（班長）を二重下線、研究分担者（班員）を一重下線、研究協力者（班長付き及び班員付き）を一重破線で示した。

Ⅳ. 研究成果の刊行物・別刷り

PLOS ONE

Suppression of La Antigen Exerts Potential Antiviral Effects against Hepatitis A Virus

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Abstract

Background: Despite the development and availability of hepatitis A virus (HAV) vaccine, HAV infection is still a major cause of acute hepatitis that occasionally leads to fatal liver disease. HAV internal ribosomal entry-site (IRES) is one of the attractive targets of antiviral agents against HAV. The aim of the present study is to evaluate the impact of La, one of the cellular proteins, on HAV IRES-mediated translation and HAV replication.

Methods and Findings: We investigated the therapeutic feasibility of siRNAs specific for cellular cofactors for HAV IRES-mediated translation in cell culture. It was revealed that siRNA against La could inhibit HAV IRES activities as well as HAV subgenomic replication. We also found that the Janus kinase (JAK) inhibitors SD-1029 and AG490, which reduce La expression, could inhibit HAV IRES activities as well as HAV replication.

Conclusions: Inhibition of La by siRNAs and chemical agents could lead to the efficient inhibition of HAV IRES-mediated translation and HAV replication in cell culture models. La might play important roles in HAV replication and is being exploited as one of the therapeutic targets of host-targeting antivirals.

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Introduction

Hepatitis A virus (HAV) is a non-enveloped single-stranded RNA virus, with ~7.6 kb positive-sense genome. The genome includes 5′ non-translated region (5′NTR), one open reading frame encoding structural (VP4, VP2, VP3, VP4 and 2A) and non-structural proteins (2B, 2C, 3A, 3B, 3C and 3D), and 3′NTR [1]. HAV genome translation could be initiated by cap-independent mechanism through HAV internal ribosomal entry-site (IRES) with a pyrimidine-rich tract, which is located at the down-stream part of 5′NTR [2]. HAV is still a major cause of acute hepatitis [3,4]. Although acute liver failure due to HAV is not common, it is still occasionally fatal [5], despite HAV vaccine having become available [6–8]. This emphasizes the importance of the development of antiviral agents against HAV.

In general, two distinct classes of antiviral agents, direct-acting antivirals (DAAs) and host-targeting antivirals (HTAs), exist [9]. Several groups have reported DAAs against HAV, such as inhibitors of HAV 3C cysteine proteinase, which is essential for viral replication and infectivity [10–15]. Small interfering RNAs against HAV genome are also varieties of DAAs [16–18]. Several broad-target HTAs, examples of which include interferon-α, interferon-β, interferon-λ1 and amantadine, have been developed and tested against HAV [2,19–25]. These compounds could inhibit HAV IRES-dependent translation as well as HAV replication [2,21,22]. HTAs of the targeted group are more precise in that they act on key host enzymes or cellular factors that are required for the viral lifecycle [9].

Our previous studies suggested that several siRNAs against HAV 5′NTR suppress HAV translation as well as HAV replication [17]. The nucleotide sequences of 5′NTR are one of the most conserved in HAV genomes [8,26]. These facts suggest that HAV IRES is one of the attractive targets of antiviral agents against HAV. It has been reported that several cellular proteins such as autoantigen La [27], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [28,29], polypyrimidine tract-binding protein (PTB/hnRNPI) [29–31], poly(C) binding protein 2 (PCBP2/hnRNP-E2) [32], polyadenylate-binding protein-1 (PABP) [33], eukaryotic translation initiation factor 4E (eIF4E) [34] and eukaryotic translation initiation factor 4E (eIF4G) [33,35,36] could interact with HAV IRES *in vitro* or *in vivo*, and could be associated with HAV replication.

Human La protein is predominantly localized in the nucleus and is associated with RNA metabolism [37]. It has been reported that La was associated with U1 RNA [38], telomerase RNA [39], 5′NTR of poliovirus [27], hepatitis C virus (HCV) [40] and GRP78/Bip [41]. La could interfere with IRES-mediated translation.

In the present study, we investigated the therapeutic feasibility of siRNAs specific for these putative cellular cofactors for HAV IRES-mediated translation. It was revealed that siRNA against La (siRNA-La) could inhibit HAV IRES activities as well as HAV subgenomic replication. We also found that JAK inhibitors SD-1029 and AG490, which inhibit La expression, could inhibit HAV IRES activities as well as HAV replication. The present study demonstrated the proof-of-concept for the inhibition of La as a method for suppressing HAV replication.

Results

Effects of silencing of cellular factors on HAV IRES-mediated translation

Although the exact mechanisms are not fully understood, it has been reported that HAV IRES could interact with various endogenous genes [27–34], suggesting important roles of these proteins in HAV IRES-mediated translation and HAV replication. La, GAPDH, PTB and PCBP2 have been shown to bind to HAV IRES domains IIIb and V [23], IIIa [28], I-IIIb [30] and I-IIIb [32], respectively. PABP, eIF4E and eIF4G also interact with HAV IRES [33–36]. To determine whether each siRNA against these factors had a specific siRNA effect, knockdown of these molecules was validated by Western blotting, respectively (Figure 1A–1G). To examine the effects of the knockdown of these genes on HAV IRES-mediated translation, Huh7 cells were cotransfected with each siRNA, and pSV40-HAV-IRES, which contains SV40 promoter, renilla luciferase (Rluc) and nt. 139–854 of HAV sequence fused firefly luciferase (Fluc) gene [2]. After 48 h transfection, the cell lysates were analyzed for HAV IRES activities (Fluc/Rluc) as previously described (Figure 1H) [17,21]. Compared with the HAV IRES activity in Huh7 cells transfected with control siRNA (siRNA-control) (100%), that transfected with siRNA-La was 39%, but those of the others were not inhibited (Figure 1H). These results provide further evidence of La being a potential cofactor for HAV IRES activity, indicating the possible usefulness of siRNA-La against HAV infection.

Effects of silencing of La on HAV subgenomic replication

Next, we examined the effect of the silencing of La on HAV subgenomic replication [42] (Figure 2). To test luciferase activity due to translation or translation and replication, we introduced a replication-competent HAV replicon (pT7-18f-LUC) and a replication-incompetent HAV replicon (pT7-18f-LUC mut) into HuhT7 cells [21,42], with or without amantadine treatment, which is effective for suppressing HAV replication [2,21]. Reporter assays were performed 24 h, 48 h or 72 h after transfection. Relative luciferase activities of pT7-18f-LUC co-transfected with siRNA-control or siRNA-La, respectively, were 100% or 15.4% at 24 h, 100% or 28.7% at 48 h, and 100% or 21.7% at 72 h after transfection (Figure 2A). On the other hand, those of pT7-18f-LUC mut cotransfected with siRNA-control or siRNA-La, respectively, were 94% or 3.7% at 24 h, 63.8% or 7.9% at 48 h, and 54.2% or 2.9% at 72 h after transfection (Figure 2A). Because the luciferase values of pT7-18f-LUC or pT7-18f-LUC mut were due to translation with replication or translation without replication, respectively [21,42], it was confirmed that siRNA-La might suppress HAV IRES-mediated translation. The effects of siRNA-La also enhanced the amantadine induced-suppression of HAV subgenomic replication (Figure 2B).

JAK inhibitors AG490 and SD-1029 could suppress La expression

Because it was reported that La expression is dependent on $JAK2^{V617F}$ in murine pro B Ba/F3-EPOR-derived cell line [42], the effects of two JAK2 inhibitors, AG490 and SD-1029, on La expression were examined. Initially, we evaluated the cytotoxicity of AG490 and SD-1029 on African green kidney GL37 cells by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. AG490 concentration in a range of 100–10,000 nM and SD-1029 concentration in a range of 100–5,000 nM were not toxic in 48-h incubation (Figure 3A, 3B). With these concentrations, we tested the effects of AG490 and SD-1029 on La expression in GL37 cells, which supports HAV replication [21]. The results of Western blotting showed that La expression was decreased in a concentration-dependent manner with AG490 (Figure 3C) and SD-1029 (Figure 3D). These data prompted us to examine whether these drugs had an inhibitory effect on HAV IRES-mediated translation or HAV replication.

Effects of AG490 and SD-1029 on HAV IRES-mediated translation

COS7 cells stably expressing pSV40-HAV-IRES (COS7-HAV-IRES cells) were generated. To evaluate HAV IRES activity, after COS7 cells were cotransfected with pSV40-HAV IRES and pCXN2, and cultured in the presence of 500 μg/mL G418 for 3 weeks, COS7-HAV-IRES cells were established, making it easy to evaluate HAV IRES activity (Figure 4A). Treatment of these cells with AG490 resulted in the inhibition of HAV IRES activities (100%, 94%, 99%, 93%, 71% and 70% at 0, 100, 500, 1,000, 5,000 and 10,000 nM AG490, respectively) (Figure 4B). Treatment of these cells with SD-1029 resulted in the inhibition of HAV IRES activities (100%, 95%, 96%, 85%, 42% and 21% at 0, 100, 500, 1,000, 5,000 and 10,000 nM SD-1029, respectively) (Figure 4C).

Effects of AG490 and SD-1029 on HAV replication

We established GL37 stably expressing both short hairpin (sh)RNA-La (GL37-shLa cells) and control shRNA (GL37-shC cells) after GL37 cells were cotransfected with plasmid shRNA-La and plasmid shRNA-control, respectively, and cultured in the presence of puromycin. We examined whether shRNA-La could inhibit the replication of HAV HA11-1299 genotype IIIA strain in these GL37-derived cell lines. Western blotting analysis demonstrated that knockdown of La was validated in GL37-shLa cells, compared to GL37-shC cells (Figure 5A). As shown in Figure 5B, HAV RNA levels were 6.07×10^5 copies/μg cellular RNA (92%) in GL37-shLa cells, in comparison with 6.63×10^5 copies/μg cellular RNA (100%) in GL37-shC cells after 72 h of HAV infection at a multiplicity of infection (MOI) of 0.1.

Next, we investigated whether AG490 or SD-1029 could inhibit the replication of HAV HA11-1299 genotype IIIA strain in GL37 cells. Cells were treated with AG490 or SD-1029 for 24 h, infected with HAV HA11-1299 genotype IIIA strain at MOI of 0.1, and washed with PBS 7 h later. After 96 h of HAV infection, cellular RNA was extracted, and HAV RNA levels were determined using real-time RT-PCR. As shown in Figure 5C, HAV RNA levels were 5.27×10^4, 5.46×10^4 or 2.58×10^4 copies/μg of cellular RNA (63%, 65% or 31%) in GL37 treated with 100, 1,000 or

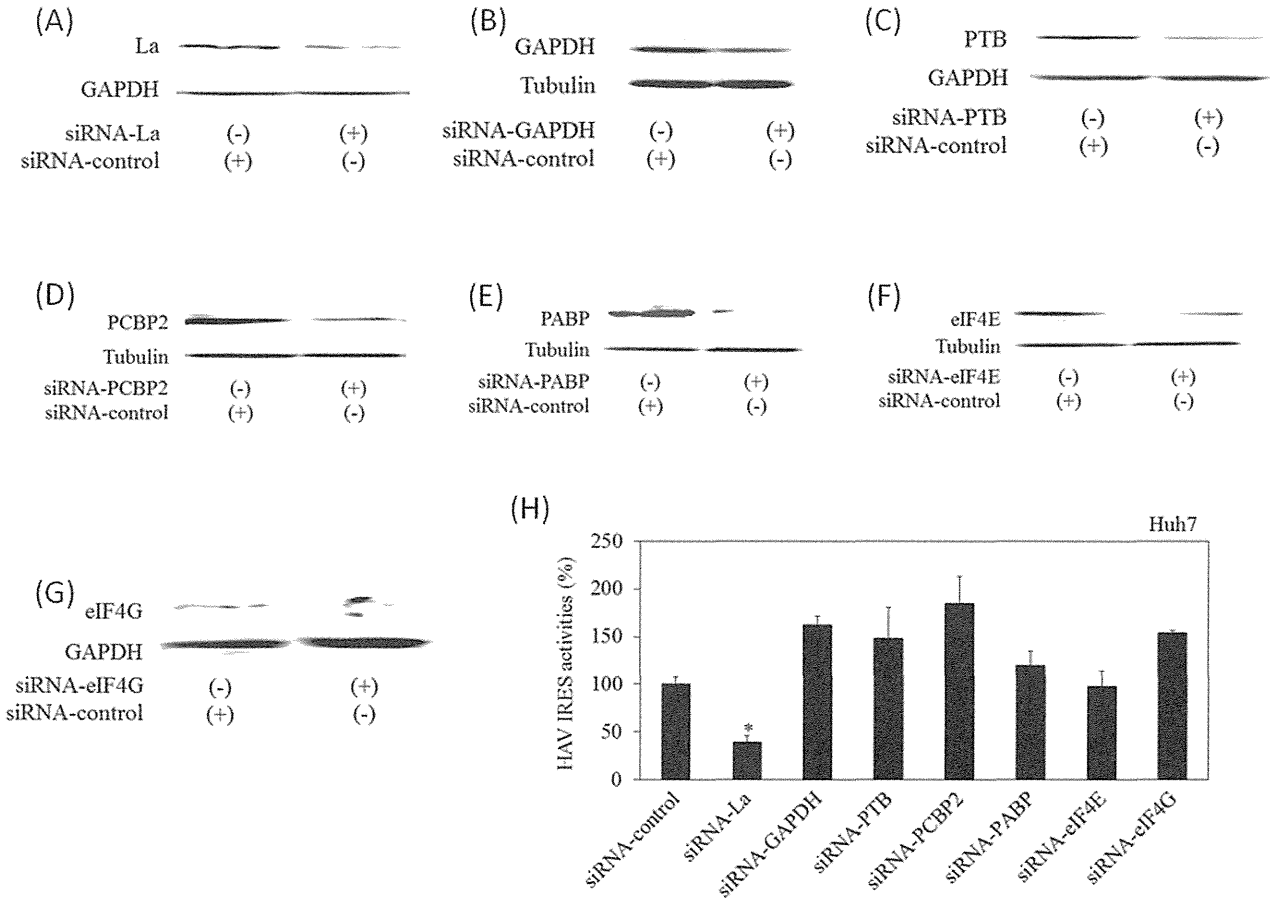

Figure 1. Knockdown of La inhibits hepatitis A virus (HAV) internal ribosomal entry site (IRES) activities. Effects of siRNAs on endogenous gene expression in Huh7 (A–G). Approximately 0.5×10^6 cells were transfected with 100 nM siRNA against La (siRNA-La), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (siRNA-GAPDH), polypyrimidine tract-binding protein (PTB/hnRNPI) (siRNA-PTB), poly(C) binding protein 2 (PCBP2/hnRNP-E2) (siRNA-PCBP2), polyadenylate-binding protein-1 (PABP) (siRNA-PABP), eukaryotic translation initiation factor 4E (eIF4E) (siRNA-eIF4E), eukaryotic translation initiation factor 4E (eIF4G) (siRNA-eIF4G) or control siRNA (siRNA-control). Protein expression was determined by Western blotting using each specific antibody. GAPDH or tubulin was used as control. (A) La, (B) GAPDH, (C) PTB/hnRNPI, (D) PCBP2, (E) PABP, (F) eIF4E, and (G) eIF4G expressions are shown. (H) Effects of each siRNA on the HAV IRES activities. Huh7 cells were cotransfected with 0.3 μg pSV40-HAV-IRES [2] with each siRNA at 100 nM. Cells were harvested 48 h post-transfection and luciferase activities were measured. Activities of HAV IRES were calculated as previously described [17,21]. Data are expressed as mean ± SD. *$P<0.05$ vs. Huh7 cells transfected with (siRNA-control).*
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10,000 nM AG490, respectively, in comparison with 8.35×10^4 copies/μg of cellular RNA (100%) in GL37 without any treatment after 96 h of HAV infection at MOI of 0.1. As shown in Figure 5D, HAV RNA levels were 4.26×10^4 or 4.12×10^4 copies/μg cellular RNA (51% or 49%) in GL37 treated with 100 or 1,000 nM SD-1029, respectively, in comparison with that in GL37 without any treatment after 96 h of HAV infection at a MOI of 0.1. ELISA analysis of tissue culture-adapted HAV KRM003 genotype IIIB strain in GL37 cells [21] also showed mild inhibition of viral propagation with 500–1,000 nM AG490 but not with SD-1029 at 48 h post-infection (data not shown).

Discussion

In the present study, we examined the effects of the knockdown of La in cell lines infected with HAV. We observed the inhibition of HAV replication by sh-La. We also observed that inhibitors of La, AG490 and SD-1029, induced the suppression of HAV genotype IIIA replication. Of course, HAV vaccine has already been developed. Although patients with acute hepatitis A are not usually treated with antiviral drugs, there are occasionally patients with severe acute hepatitis A such as fatal acute liver failure. To our knowledge, ours is the first study to report that a reduction of La can suppress HAV replication in cell culture.

It has been reported that down-regulation of La was induced by (-)-epigallocatechin gallate, iron chelator deferxamine and JAK inhibitor AZD1480 [43–45]. Ferric ammonium citrate up-regulates La expression [45]. It was reported that HBSC-11, an inhibitor of La, has an anti-HBV activity in which HBSC-11 may be mediated by a reduction in La levels [46]. Because we did not observe any effects of (-)-epigallocatechin gallate or ferric ammonium citrate on La expression in our experiments (data not shown), we chose JAK inhibitors in the present study. Our study suggested that anti-HAV activity of AG490 and SD-1029 should also be mediated by a reduction of La. It is possible that La inhibitors could be useful as antiviral drugs.

The use of 1,000 nM of AG490 and SD-1029 reduced La expression in GL37 cells (Figure 3C, 3D). However, there were no effects on HAV IRES activities up to this concentration in COS7-

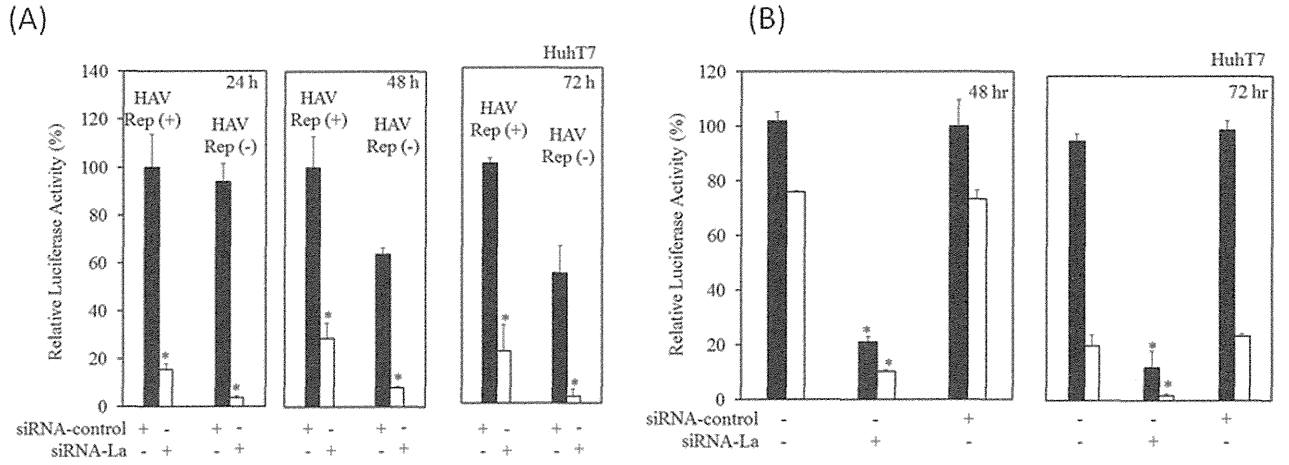

Figure 2. Knockdown of La inhibits hepatitis A virus (HAV) subgenomic replication. (A) Effects of siRNA against La (siRNA-La) on the HAV *replication-competent replicon* pT7-18f-LUC [HAV Rep (+)] or *replication-incompetent replicon* pT7-18f-LUCmut [HAV Rep (−)] replication. Approximately 0.5×10^6 HuhT7 cells were cotransfected with 0.3 μg pT7-18f-LUC/pT7-18f-LUCmut [42] and 100 nM siRNA against La (siRNA-La)/control siRNA (siRNA-control). After 24 h (left), 48 h (middle) or 72 h (right), cell lysates were collected and luciferase activities were measured. (B) Effects of siRNA against La (siRNA-La) with or without amantadine on the HAV subgenomic replication-competent replicon pT7-18f-LUC. Approximately 0.5×10^6 HuhT7 cells were cotransfected with 0.3 μg pT7-18f-LUC and 50 nM siRNA against La (siRNA-La)/control siRNA (siRNA-control). After 24 h transfection, cells were treated with 5 μg/mL amantadine (white column) or without (black column). After 48 h (left) or 72 h (right) transfection, cell lysates were collected and luciferase activities were measured. Data are expressed as mean ± SD. *$P<0.05$.
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HAV-IRES cells (Figure 4B, 4C). These discrepancies might be a result of these two different cell lines, or this might be one of the points needing improvement in COS7-HAV-IRES cells.

Although AZD1480 was an inhibitor of JAK1 and JAK2 [46], AG490 is a tyrosine kinase inhibitor of JAK2, JAK3, epidermal growth factor (EGFR) and v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (Neu) [47,48], and JAK2 inhibitor III SD-1029 acts as a JAK2-selective inhibitor [49]. Of interest is that these three JAK inhibitors reduce cellular La expression (Figure 3C and 3D) [45]. HAV and HCV modulate the JAK/STAT signaling pathway [50,51]. Further studies will be needed at this stage, although several specific JAK inhibitors have been developed and

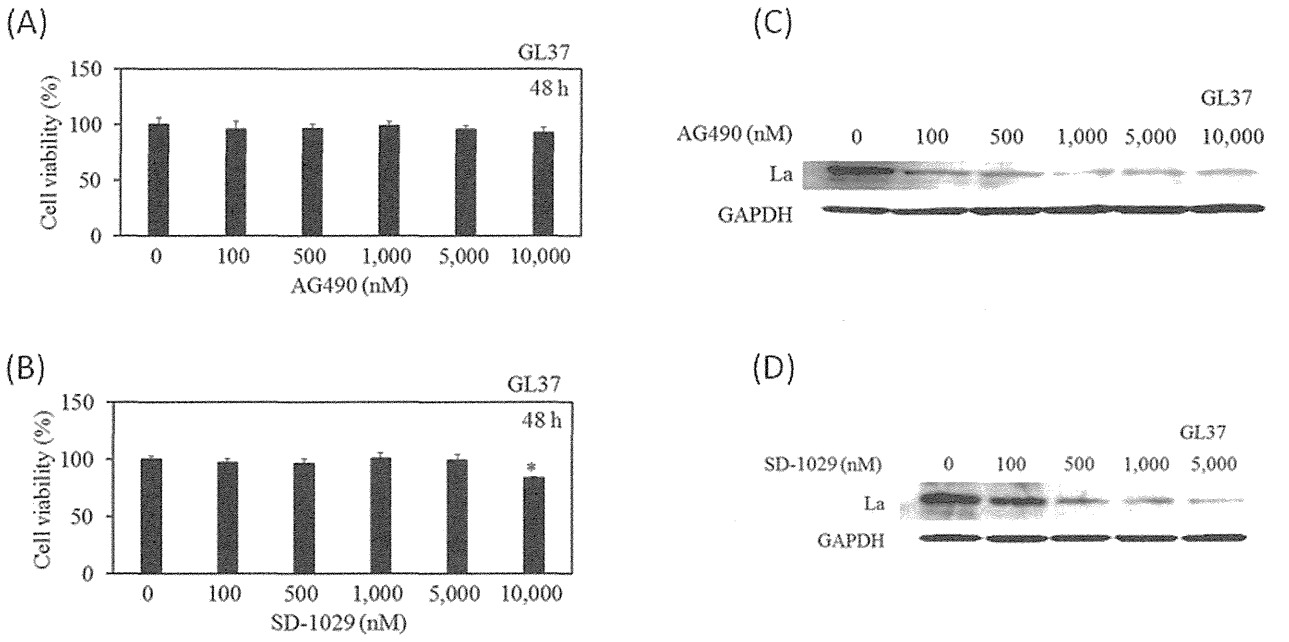

Figure 3. Effects of AG490 and SD-1029 on cell viability and La expression in GL37 cells. MTS assays of cells 48 h after treatment with AG490 (A) or SD-1029 (B) at indicated concentrations. Data are expressed as mean ± SD. Western blotting analysis. Approximately 1×10^5 cells were incubated in the presence of AG490 (C) or SD-1029 (D) at indicated concentrations. Twenty-four hours after treatment, cell lysates were analyzed for La and GAPDH expressions using specific antibodies. Data are expressed as mean ± SD. *$P<0.05$.
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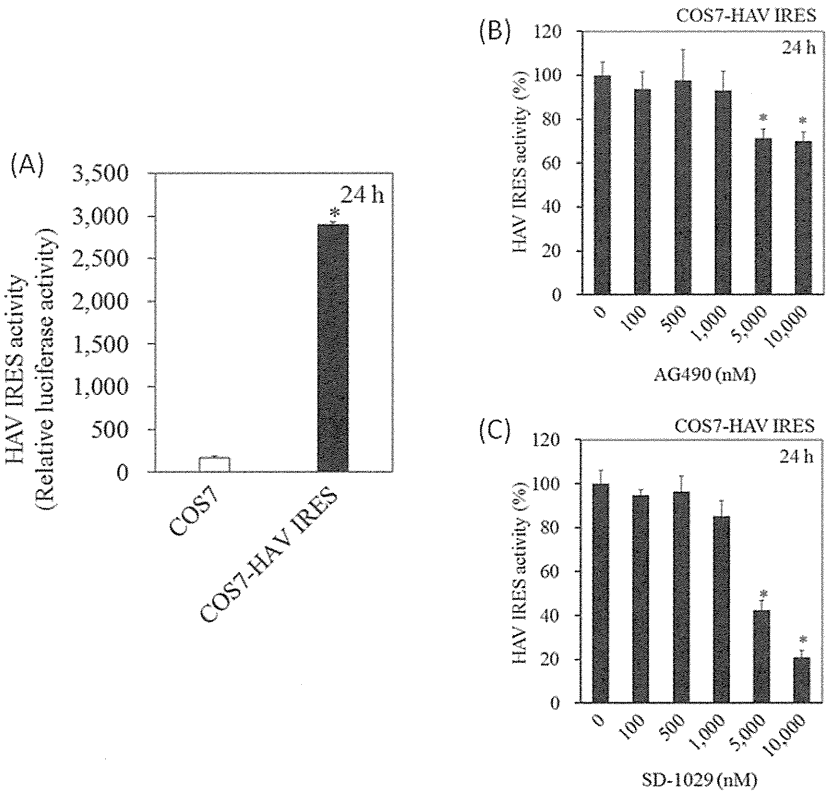

Figure 4. Effects of AG490 and SD-1029 on hepatitis A virus (HAV) internal ribosomal entry site (IRES) activities. (A) HAV IRES activities of COS7 cells stably expressing pSV40-HAV-IRES (COS7-HAV IRES cells). 0.5×10^5 cells were seeded, cell lysates were collected 24 h later, and luciferase assay was performed for determination of HAV IRES activities. (B) Effect of AG490 on HAV IRES activity in COS7-HAV IRES cells. (C) Effect of SD-1029 on HAV IRES activity in COS7-HAV IRES cells. The cells were cultured with AG490 or SD-1029 at the concentrations indicated, and reporter assay was performed after 24 h of treatment. Activities of HAV IRES were calculated as previously described [17,21]. Data are expressed as mean ± SD. *$P<0.05$.
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there are ongoing trials for the treatment of myeloproliferative neoplasms [52], allergic skin diseases [53] and rheumatoid arthritis [54].

HAV replicates in the cytoplasm of hepatocytes, although La exists predominantly in the nucleus [37]. Previous studies have suggested that La associates with IRES-mediated translation [27,40,41], and the present study also demonstrated that La plays a potential role in HAV IRES-mediated translation. Our result also showed that HAV IRES-mediated translation was helped by La, in contrast to the previous observation [27]. These differences might be related to the experimental system such as in vivo or in vitro, and cell lines. Although HAV RNA levels were reduced by 5.6×10^4 copies/μg cellular RNA when comparing GL37-shLa cells with GL37-shC cells (Figure 5B), it might be possible that other host factors are involved in suppressing HAV replication by JAK inhibitors. Further studies will be needed.

In the study field of HCV infection, the development of two distinct antiviral agents, DAAs and HTAs, could lead to higher sustained virological response rates via reductions of adverse events and treatment duration [9] compared to the former standard treatment [55]. The use of La inhibitor, one of the HTAs for HAV, alone or in combination with DAAs, might be beneficial for certain patients infected with HAV.

There are three HAV genotypes, I, II and III, of human origin [56]. The inhibitory effects of AG490 and SD-1029 on HAV subgenotype IIIA strain were observed by real-time PCR methods. But only weak inhibition of AG490 on HAV subgenotype IIIB was observed by ELISA methods. This may be related to the different methods of detection for HAV, that is, by RT-PCR or ELISA. There might also be differences among the different HAV subgenotypes, although HTAs have a high genetic barrier to resistance and a pan-genotypic antiviral activity [57]. Further studies on the exact mechanism of the association between La and HAV replication will be needed. In conclusion, inhibition of La by siRNAs and chemical agents could lead to the inhibition of HAV IRES-mediated translation and HAV replication in cell culture models. Our findings suggest that La plays important roles in HAV replication and should be exploited as one of the therapeutic targets.

Materials and Methods

Cells, virus and reagents

Human hepatoma cells (Huh7 and HuhT7, which stably express T7-RNA polymerase [42]) and African green monkey kidney cells (COS7 and GL37 [21,22,56,58]), were grown in Dulbecco's modified essential medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma) at 37°C in 5% CO_2. Huh7 and COS7 cells were purchased from JCRB cell bank, National Institute of Biomedical Innovation, Osaka, Japan. HAV subgenomic replicon was previously described [42]. Briefly, the structures of the competent HAV replicon (HAV) and incompetent HAV replicon (mut-HAV replicon) containing a

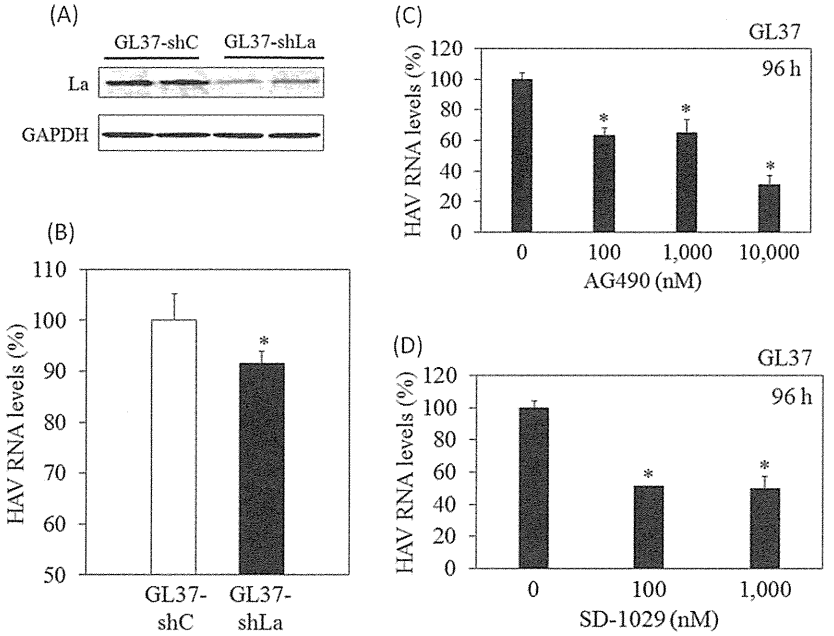

Figure 5. Antiviral activities of shRNA-La, AG490 and SD-1029 on hepatitis A virus (HAV) replication. (A) La protein expression in GL37 stably expressing shRNA-La (GL37-shLa cells) and GL37 stably expressing control shRNA (GL37-shC cells). Western blotting was performed with specific antibodies indicated. (B) Real-time PCR analysis of intracellular HAV RNA following HAV HA11-1299 genotype IIIA strain infection in GL37-shC or GL37-shLa cells. (C) Suppression of HAV infection in GL37 treated with AG490 at concentrations indicated. (D) Suppression of HAV infection in GL37 treated with SD-1029 at concentrations indicated. Cells were treated with AG490 or SD-1209 for 24 h, infected with HAV HA11-1299 genotype IIIA strain at MOI of 0.1, and washed with PBS 7 h later. After 96 h of HAV infection, cellular RNA was extracted, and HAV RNA levels were determined using real-time RT-PCR. Data are expressed as mean ± SD. *$P<0.05$.
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frame-shift mutation in polymerase 3D were reported [42]. These replicons also contain an open-reading frame of firefly luciferase. HAV HA11-1299 genotype IIIA strain and HAV KRM003 genotype IIIB strain [21] were also used. The simian virus (SV) 40 promoter plasmid pSV40-HAV IRES was used as reported previously [2,17]; it encodes in a bicistronic fashion the Renilla reniformis luciferase (Rluc), the hepatitis A virus internal ribosomal entry site (HAV IRES), followed by the firefly luciferase (Fluc). AG490 (Calbiochem, Billerica, MA, USA), SD-1029 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and amantadine (Sigma) were used in the present study.

Transfection of shRNA and siRNAs

To stably establish GL37-shLa and control GL37-shC cells, respectively, we used the plasmids shRNA-La (shLa) and shRNA-control (shC) purchased from Santa Cruz. After electroporation of the plasmids, GL37 cells were placed in 10-mm-well plates (Iwaki Glass, Tokyo, Japan), and treated with 3 μg/mL puromycin for selection of antibiotic-resistant colonies over a 2-week period. We then confirmed the expression of endogenous La by Western blotting. siRNAs against La (siRNA-La), GAPDH (siRNA-GAPDH), PTB (siRNA-PTB), PCBP2 (siRNA-PCBP2), PABP (siRNA-PABP), eIF4E (siRNA-eIF4E), eIF4G (siRNA-eIF4G) and control siRNA (siRNA-control) were purchased from Santa Cruz.

Transfection and luciferase assay

Huh7 cells were seeded in 6-well plates one day before transfecton, and cotransfected with 0.3 μg of the pSV40-HAV-IRES plasmid and 100 nM of each siRNA using Effectene transfection reagent (Qiagen, Hilden, Germany). Forty-eight hours after transfection, cell lysates were collected using a luciferase lysis buffer (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was measured with a luminometer (AB-2200-R; ATTO, Tokyo, Japan).

Western blotting

Cells were lysed in sodium dodecyl sulfate sample buffer, and after sonication, lysates were used for Western blotting analysis. Briefly, proteins were subjected to electrophoresis on 5–20% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (ATTO, Tokyo, Japan). Membranes were incubated with specific antibodies for La, GAPDH, PTB, PCBP2, PABP, eIF4E, eIF4G and tubulin (Santa Cruz). After washing, membranes were incubated with secondary horse-radish peroxidase–conjugated antibodies. Signals were detected by means of enhanced chemiluminescence (GE Healthcare, Tokyo, Japan) and scanned by image analyzer LAS-4000 and Image Gauge (version 3.1) (Fuji Film, Tokyo, Japan) and Scion Image (Scion) software.

RNA extraction and real-time RT-PCR

After 96 h or 72 h of HAV infection, total RNA was isolated using the RNeasy Mini Kit (Qiagen). One microgram of RNA was reverse-transcribed with the PrimeScript RT reagent (Perfect Real Time; Takara, Otsu, Japan). PCR amplification was performed on cDNA templates using primers specific for HAV (sense primer 5′-AGGCTACGGGTGAAACCTCTTAG-3′ and antisense primer 5′-GCCGCTGTTACCCTATCCAA-3′) [59]. For RNA quantification, real-time PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, Forester City, CA, USA) following the manufacturer's protocol. Data analysis was based on the Standard curve method.

MTS assay

To evaluate cell viability, MTS assays were performed using a Cell Titer Aqueous One Solution Proliferation Assay (Promega) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using Student's t-test. *P*-values <0.05 were considered statistically significant.

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Author Contributions

Conceived and designed the experiments: XJ T. Kanda SW OY. Performed the experiments: XJ T. Kanda SW SN KS HS T. Kiyohara KI TW HO. Analyzed the data: XJ T. Kanda SW HS T. Kiyohara KI TW HO OY. Contributed reagents/materials/analysis tools: HS T. Kanda KI OY. Contributed to the writing of the manuscript: XJ T. Kanda T. Kiyohara KI TW HO OY.

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The JAK2 inhibitor AZD1480 inhibits hepatitis A virus replication in Huh7 cells

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ABSTRACT

The JAK2 inhibitor AZD1480 has been reported to inhibit La protein expression. We previously demonstrated that the inhibition of La expression could inhibit hepatitis A virus (HAV) internal ribosomal entry-site (IRES)-mediated translation and HAV replication *in vitro*. In this study, we analyzed the effects of AZD1480 on HAV IRES-mediated translation and replication. HAV IRES-mediated translation in COS7-HAV-IRES cells was inhibited by 0.1–1 μM AZD1480, a dosage that did not affect cell viability. Results showed a significant reduction in intracellular HAV HA11-1299 genotype IIIA RNA levels in Huh7 cells treated with AZD1480. Furthermore, AZD1480 inhibited the expression of phosphorylated-(Tyr-705)-signal transducer and activator of transcription 3 (STAT3) and La in Huh7 cells. Therefore, we propose that AZD1480 can inhibit HAV IRES activity and HAV replication through the inhibition of the La protein.

1. Introduction

Hepatitis A virus (HAV) infection is a major cause of acute hepatitis in both developing and developed countries [1–7]. In developed countries, persons hospitalized for hepatitis A tend to be older and are more likely to have other liver diseases and/or other comorbid medical conditions [7,8]. HAV belongs to the *Picornaviridae* family and possesses an internal ribosomal entry-site (IRES) that is a responsible for its cap-independent translation initiation. Among picornaviruses, only HAV and poliovirus can be controlled with vaccinations [9]. However, the costs are relatively expensive, and vaccinations are not universal in some countries, including Japan [10]. Despite the availability of efficient HAV vaccines, anti-HAV drugs are required to treat severe cases such as acute liver failure, outbreak cases, and vaccine-escape variants [11].

Recently, we reported that the Janus kinase (JAK) inhibitors SD1029 and AG490 reduced La expression and inhibited HAV IRES activities and HAV replication [12]. In the present study, two different antiviral assays were used: (i) inhibition of HAV IRES activity assay using COS7 cells stably expressing the HAV IRES reporter, and (ii) inhibition of HAV genotype IIIA replication in the human hepatoma cell line Huh7. We examined whether another JAK2 inhibitor (AZD1480) could inhibit HAV IRES activity and HAV replication. We also examined the effects of AZD1480 on the expression of phosphorylated-(Tyr-705)-signal transducer and activator of transcription 3 (STAT3) and La.

2. Materials and methods

2.1. Cell lines and reagents

The African green monkey kidney cell line COS7 and the human hepatoma cell line Huh7 were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma–Aldrich, St. Louis, MO, USA) under 5% CO_2 at 37 °C. The cultures were supplemented with AG490 (Calbiochem, Billerica, MA, USA), SD1029 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), AZD1480 (Selleck

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Chemicals, Houston, TX), interferon α-2a (Sigma–Aldrich), and amantadine (Sigma–Aldrich) where indicated.

2.2. RNA extraction and quantification of HAV RNA

Total cellular RNA was extracted from harvested cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 0.5 μg of total RNA using the PrimeScript RT reagent (Perfect Real Time; Takara, Otsu, Japan). Reverse transcription was performed at 37 °C for 15 min, followed by 95 °C for 5 s. For HAV RNA quantification, the following primer set was used: sense primer, 5′-AGGCTACGGGT-GAAACCTCTTA-3′ and antisense primer, 5′-GCCGCTGTTACCCTATC-CAA-3′ [13]. The primer set for the quantification of GAPDH mRNA was previously described [12]. Real-time PCR was performed with SyBr Green I on a StepOne Real-Time PCR system (Applied Biosystems). The PCR reaction was performed as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data analysis was based on the ΔΔCt method. Specificity was validated using melting curve analysis.

2.3. Western blot

The cells were lysed using sodium dodecyl sulfate lysis buffer. The proteins were subjected to electrophoresis on a 5–20% polyacrylamide gel and transferred onto a nitrocellulose membrane (ATTO, Tokyo, Japan). The membrane was probed with an antibody against phosphorylated-(Tyr-705)-STAT3, STAT3 (Cell Signaling Technology, Danvers, MA, USA), La or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The proteins were visualized using an enhanced chemiluminescent ECL Western blot substrate (GE Healthcare, Tokyo, Japan).

2.4. Infection of Huh7 cells with HAV

Huh7 cells were seeded 24 h before infection at a density of 1×10^5 cells/well in 12-well plates (AGC Techno Glass, Shizuoka, Japan). The cells were washed twice with PBS and infected with the HAV HA11-1299 genotype IIIA strain at a multiplicity of infection (MOI) of 0.1 in DMEM containing 2% FBS [12]. After 24 h of incubation, the cells were washed three times with PBS, followed by the addition of 1 mL of DMEM containing 2% FBS. After 72 or 96 h of incubation, the levels of HAV RNA in the inoculated cells were determined using real-time RT-PCR.

2.5. Luciferase assay

The SV40-HAV-IRES plasmid was constructed to analyze HAV IRES-mediated translation efficacy [14]. This HAV IRES was derived from pHM175 (kindly provided by Professor Suzanne U. Emerson, National Institutes of Health, MD, USA). Briefly, a plasmid expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HAV IRES-mediated translation initiation, and the pCXN2 vector (kindly provided by Professor Junichi Miyazaki, Osaka University, Japan) harboring a neomycin-resistant gene [15] were introduced by electroporation (850 μF and 250 V) into 5×10^6 COS7 cells using the Bio-Rad Gene Pulser Xcell system (Hercules, CA, USA). After 2 weeks of treatment with 1000 μg/mL G418 (Promega, Madison, WI, USA), COS7-HAV-IRES cells were cloned and established.

For the detection of HAV IRES activity, 10,000 COS7-HAV-IRES cells/well were seeded into a 96-well plate with or without various reagents as indicated. Forty-eight hours later, the cells were harvested using reporter lysis buffer (Toyo Ink, Tokyo, Japan) and luciferase activities were determined using a luminometer (Luminescencer-JNR II AB-2300, ATTO, Tokyo, Japan). All samples were run in triplicate.

2.6. MTS assays

For the evaluation of cell growth and cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using the CellTiter 96 Aqueous One-Solution cell proliferation assay (Promega). Enzyme activity was measured with a Bio-Rad iMark microplate reader (Bio-Rad) at the 490 nm wavelength.

2.7. Statistical analysis

Data are expressed as the mean ± standard deviations (SD). Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of JAK2 inhibitors on COS7-HAV-IRES cell viability

To evaluate the effect of JAK inhibitors AZD1480, SD1029 and AG490 on HAV IRES activity, 5000 COS7-HAV-IRES cells per well were incubated with the inhibitors for 48 h (Fig. 1A–C). Interferon α-2a and amantadine were used as positive controls (Fig. 1D and E). The cytotoxicity of the drugs against COS7-HAV-IRES cells was determined using the MTS assay. We observed that the cell viabilities were not affected by supplementation with 0.1–1 μM AZD1480, 0.01–1 μM SD1029, 0.01–10 μM AG490, 1000–10,000 U/mL interferon α-2a and 0.5–50 μg/mL amantadine (Fig. 1A–E). These results showed that AZD1480 concentrations equal to or below 1 μM were safely tolerated by the cells.

3.2. Inhibitory effects of JAK2 inhibitors on HAV IRES activity in COS7-HAV-IRES cells

We previously reported that SD1029 and AG490 could inhibit HAV IRES activity and HAV replication in the African green monkey kidney cell line GL37 [12,16]. In the present study, we examined whether AZD1480 could inhibit HAV IRES activity in COS7-HAV-IRES cells. In COS7-HAV-IRES cells treated with 0.1 and 1 μM AZD1480 for 48 h, HAV IRES activities were reduced to 52.2% and 44.6% of the untreated control (Fig. 1A). Similarly, in COS7-HAV-IRES cells treated with 0.01, 0.1 and 1 μM SD1029 or 0.01, 0.1, 1 and 10 μM AG490 for 48 h, HAV IRES activities were reduced to 83.1%, 83.6% and 76.5%, or 88.8%, 86.4%, 78.8% and 77.1% of the untreated control, respectively (Fig. 1B and C). In COS7-HAV-IRES cells treated with 1000 and 10,000 U/mL interferon α-2a or 0.5, 5 and 50 μg/mL amantadine for 48 h, HAV IRES activities were reduced to 55.2% and 49.4% or 54.3%, 55.0% and 50.5% of the untreated control, respectively (Fig. 1D and E). These results indicated that AZD1480 could inhibit HAV IRES-mediated translation.

3.3. Inhibition of the replication of the HAV HA11-1299 genotype IIIA strain by AZD1480

To verify whether AZD1480 could also interfere with full-length HAV replication, Huh7 cells were infected with the HAV HA11-1299 genotype IIIA strain at an MOI of 0.1 24 h after treatment with 0.1 μM or 1 μM of AZD1480. At 96 h post-infection, intracellular HAV RNA levels were reduced to 86.1 ± 7% ($n = 3$, $p = 0.050$) or 83.6 ± 5.6% ($n = 3$, $p = 0.030$) of the untreated control, respectively

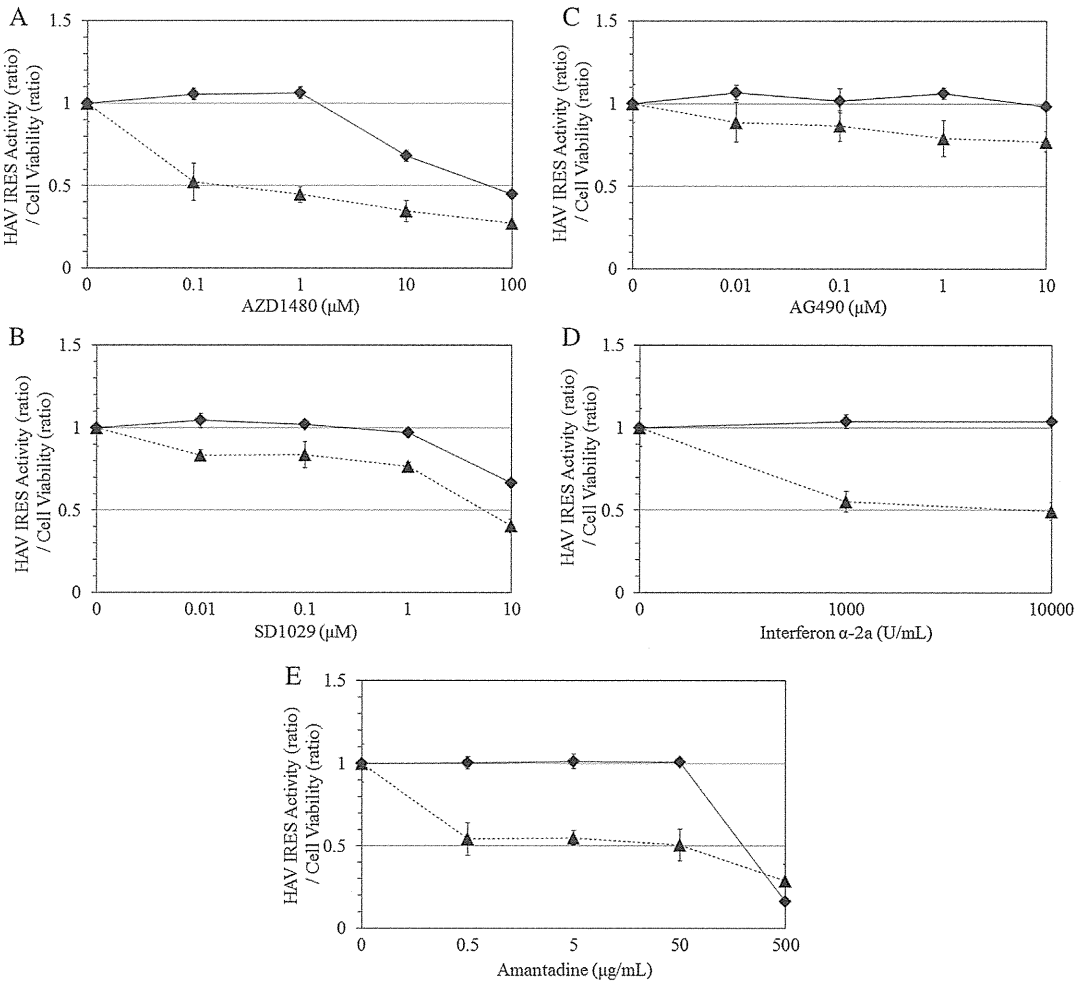

Fig. 1. Effects on cell viability and hepatitis A (HAV) internal ribosomal entry-site (IRES) activity in COS7-HAV-IRES cells. (A) AZD1480, (B) SD1029, (C) AG490, (D) interferon α-2a, (E) amantadine. Cell viability (black diamonds) was evaluated using the MTS assay (Promega). HAV IRES activities (black triangles) were evaluated as previously described [12].

(Fig. 2). At 72 h post-infection, HAV RNA levels in cells treated with1 μM AZD1480 were reduced to 91.1 ± 4.6% (n = 3, p = 0.033) of the untreated control. These results showed that AZD1480 could inhibit HAV replication in the human hepatoma cell line Huh7.

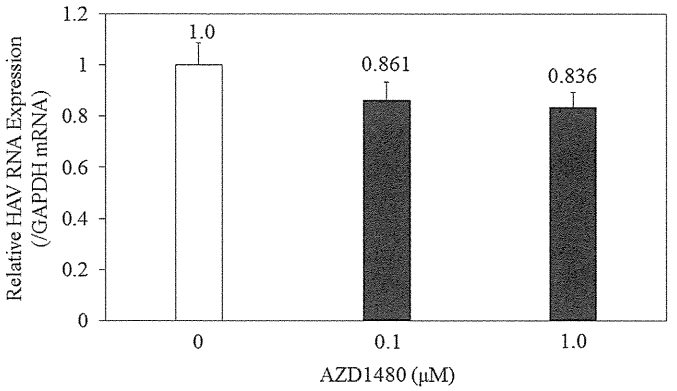

Fig. 2. Inhibition of HAV HA11-1299 genotype IIIA strain replication by AZD1480 in Huh7 cells. Huh7 cells were infected with the HAV HA11-1299 genotype IIIA strain at an MOI of 0.1 24 h after treatment with 0.1 μM or 1 μM AZD1480. At 96 h post-infection, intracellular HAV RNA levels were evaluated by real-time RT-PCR. The data are expressed as means ± standard deviations (SD).

3.4. Effects of AZD1480 on STAT3 and La protein expression in Huh7 cells

To further explore the mechanism behind the above results, we examined the expression of the phosphorylated-(Tyr-705)-STAT3, STAT3 and La proteins in Huh7 cells treated with or without 0.1 μM or 1 μM AZD1480 (Fig. 3). The results showed that AZD1480 inhibited the expression of phosphorylated-(Tyr-705)-STAT3 and

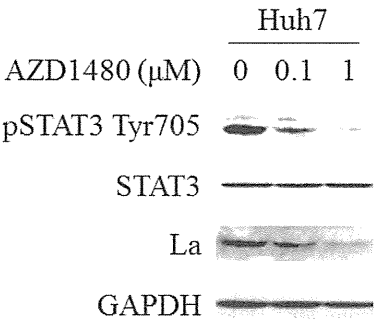

Fig. 3. Effects of AZD1480 on STAT3 and La expression in Huh7 cells. Forty-eight hours after treatment with or without AZD1480, cell lysates were analyzed for phosphorylated-(Tyr-705)-STAT3, STAT3, La and GAPDH expression using specific antibodies.

La in Huh7 cells, supporting the previous observation that AZD1480 could inhibit the La protein [17].

4. Discussion

HAV IRES-mediated translation and HAV replication are essential steps during HAV infection. We previously demonstrated that HAV IRES-mediated translation is an important target of anti-HAV treatments, resulting in the inhibition of HAV replication [12,14,18–21]. In the present study, we demonstrated that the JAK2 inhibitor AZD1480 could inhibit HAV IRES activity in addition to HAV replication. AZD1480 also inhibited the expression of phosphorylated-(Tyr-705)-STAT3 and La in Huh7 cells.

Our previous study showed that the inhibition of La by JAK inhibitor SD1029 or AG490 led to the efficient inhibition of HAV IRES-mediated translation and HAV replication in the African green monkey kidney cell line GL37 [12]. In the present study, AZD1480 in addition to SD1029 and AG490 led to the efficient inhibition of HAV IRES-mediated translation and HAV replication in Huh7 cells. Nakatake et al. reported that the V617F JAK2 mutation affected p53 response to DNA damage through the upregulation of La antigen and the accumulation of MDM2 in myeloproliferative neoplasma [17]. The authors also showed that AZD1480 inhibited the La protein.

AZD1480 inhibited the expression of phosphorylated-(Tyr-705)-STAT3 as well as the La protein in Huh7 cells. Therefore, the inhibition of the La protein might be one of the mechanisms by which HAV IRES-mediated translation and HAV replication are inhibited [12].

Waris et al. reported the constitutive activation of STAT-3 in a liver biopsy from an HCV-infected patient and suggested a potential role for STAT-3 in HCV RNA replication [22]. Inhibition of the expression of phosphorylated-(Tyr-705)-STAT3 may lead to the inhibition of HAV replication. Because several reports have demonstrated a role for STAT3 in viral replication [23–25], further studies are required to address this issue.

Two methods exist for the use of the HAV vaccine. One is a universal vaccination program, while the other is post-exposure prophylaxis. The national guidelines for hepatitis A control in Australia changed its recommendation to include the use of the hepatitis A vaccine instead of normal human immune globulin for post-exposure prophylaxis [26]. Additionally, anti-HAV drugs that prevent severe HAV infections and promote HAV eradication might contribute to post-exposure prophylaxis.

Among hepatitis A patients, patients with liver disease were hospitalized longer. Moreover, these patients had increased secondary comorbid discharge diagnoses such as liver disease, hypertension, ischemic heart disease, disorders of lipid metabolism and chronic kidney disease [7]. Thus, the availability of an anti-HAV drug would be of clinical importance [11]. In conclusion, AZD1480 significantly inhibited HAV HA11-1299 genotype IIIA strain replication *in vitro*. However, the precise mechanism of the inhibitory effect of AZD1480 was not established. Further studies are required to elucidate the mechanism.

Conflict of interest

None.

Acknowledgments

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Transparency document

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ORIGINAL ARTICLE

The complete genomes of subgenotype IA hepatitis A virus strains from four different islands in Indonesia form a phylogenetic cluster

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Abstract Despite the high endemicity of hepatitis A virus (HAV) in Indonesia, genetic information on those HAV strains is limited. Serum samples obtained from 76 individuals during outbreaks of hepatitis A in Jember (East Java) in 2006 and Tangerang (West Java) in 2007 and those from 82 patients with acute hepatitis in Solo (Central Java), Denpasar on Bali Island, Mataram on Lombok Island, and Makassar on Sulawesi Island in 2003 or 2007 were tested for the presence of HAV RNA by reverse transcription PCR with primers targeting the VP1-2B region (481 nucleotides, primer sequences at both ends excluded). Overall, 34 serum samples had detectable HAV RNA, including at least one viremic sample from each of the six regions. These 34 strains were 96.3-100 % identical to each other and formed a phylogenetic cluster within genotype IA. Six representative HAV isolates from each region shared 98.3-98.9 % identity over the entire genome and constituted a IA sublineage with a bootstrap value of 100 %, consisting of only Indonesian strains. HAV strains recovered from Japanese patients who were presumed to have contracted HAV infection while visiting Indonesia were closest to the Indonesian IA HAV strains obtained in the present study, with a high identity of 99.5-99.7 %, supporting the Indonesian origin of the imported strains. These results indicate that genetic analysis of HAV strains indigenous to HAV-endemic countries, including Indonesia, are useful for tracing infectious sources in imported cases of acute hepatitis A and for defining the epidemiological features of HAV infection in that country.

Introduction

Hepatitis A virus (HAV) is an important causative agent of acute hepatitis in humans worldwide, and is transmitted primarily through the fecal-oral route by the consumption of contaminated food and water or by person-to-person contact, but it is rarely transmitted parenterally or sexually [9, 18]. Infection with HAV is endemic in developing

Deceased: R. Amirudin.

The nucleotide sequences of HAV isolates reported herein have been assigned DDBJ/EMBL/GenBank accession nos. AB839692-AB839697 (complete genomes) and AB839698-AB839731 (481-nt VP1-2B sequences).

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countries, and the majority of individuals in these countries are exposed to HAV during early childhood. In contrast, the adult population in industrialized countries, including Japan, has had a decreasing exposure rate to HAV due to improvements in hygiene and sanitation conditions [12].

HAV is a member of the genus *Hepatovirus* within the family *Picornaviridae* [15] and contains a positive-sense, single-stranded RNA genome of approximately 7.5 kilobases (kb) in length, with a single long open reading frame (ORF). The ORF of 2227 amino acids (aa) is organized into three functional regions termed P1, P2 and P3. P1 encodes the capsid polypeptides VP1-VP4, whereas P2 and P3 encode non-structural polypeptides. The ORF is preceded by a 5′ untranslated region (UTR) and is followed by a 3′UTR with a short poly(A) tail [9]. Although HAV displays only a single serotype, HAV strains isolated from different parts of the world have been classified into six genotypes (I to VI), of which genotypes I, II and III are found in humans. These are further divided into subgenotypes IA and IB, IIA and IIB, and IIIA and IIIB, respectively [17]. Genotype I is the most prevalent worldwide, and subgenotype IA is more common than IB. Currently, the complete or nearly complete nucleotide sequence is available for at least 50 human HAV isolates of subgenotypes IA, IB, IIA, IIB, IIIA and IIIB [3, 5, 7, 8, 17, 20, 27], but there is limited or no information about the isolates in many developing countries where HAV is endemic.

In Asian countries, there is considerable variety in the seroprevalence of HAV infection, with some continuing to have high rates and others making a transition to moderate or low rates [36]. In Indonesia, one of the largest archipelagos in the world, consisting of more than 17,000 islands, a study from 1978 to 1981 found a very high seroprevalence rate, reaching a level of 95 % by the age of 10 years [2]. In studies in the mid-1990s, some urban communities had a moderate seroprevalence rate, while rural areas continued to experience a high rate [6, 13, 37].

Despite the high endemicity of HAV in Indonesia, there are scarce data on the HAV strains circulating in this country: only partial sequences of HAV strains in Bali are available [37]. Virtually nothing is known about the relatedness of HAV isolates from this country and those from other parts of the world, especially other Asian countries. This knowledge is important for establishing evolutionary relationships and information about HAV transmission events. This study presents the first molecular characterization of full-length HAV genomes in six cities on four islands in Indonesia and demonstrates the usefulness of genome analysis of HAV strains for tracing infectious sources in patients with hepatitis A imported from Indonesia.

Materials and methods

Serum samples

Serum samples were obtained from 76 individuals during outbreaks of acute hepatitis A for the purpose of screening for ongoing HAV infections in Jember (n = 7) on East Java in 2006 and Tangerang (n = 69) on West Java in 2007 (Fig. 1). In addition, serum samples were obtained from 44 patients, including 19 patients in Solo on Java Island, 17 patients in Mataram on Lombok Island, and eight patients in Makassar on Sulawesi Island, who were clinically diagnosed to have acute hepatitis in 2007. Serum samples obtained from 38 patients in Denpasar on Bali Island who had developed sporadic acute hepatitis in 2003 were also analyzed: there were no overlaps in patients between the present study and the previous study [37]. Serum samples from hepatitis patients were obtained at the first visit, and all serum samples were stored first at −20 °C in Indonesia and then at −80 °C after having been sent to Jichi Medical University, Japan, until testing. Hepatitis patients exhibited an acute illness, presenting with clinical signs or symptoms such as jaundice, dark urine, general fatigue, anorexia, nausea, vomiting and fever, and had a serum alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) level that was at least two and half times the upper limit of the normal level. Autoimmune liver disease was not excluded by the routine test. This study was approved by the Ethics Committee of the hospital, and tested individuals gave informed consent.

Serum samples were tested for IgM antibodies against HAV (anti-HAV IgM) by a chemiluminescence immunoassay (Abbott Japan, Tokyo, Japan); hepatitis B surface antigen (HBsAg) by the hemagglutination method (Mycell, Institute of Immunology Co. Ltd., Tokyo, Japan); hepatitis B virus (HBV) DNA by nested polymerase chain reaction (PCR) with primers targeting the S gene region [28]; and the IgM class of anti-HBc antibody by ELISA according to the previously described method [26]. Antibodies to hepatitis C virus (HCV) were assayed by the hemagglutination method (Fujirebio, Tokyo, Japan), and serum samples with anti-HCV antibodies were assayed for HCV RNA by reverse transcription (RT)-PCR using primers derived from well-conserved areas of the 5' UTR of the HCV genome [23]. The IgG class of antibodies against hepatitis delta virus (HDV) was assayed using an in-house enzyme-linked immunoassay (ELISA) [10]. The IgM and IgA classes of antibodies against hepatitis E virus (HEV) were also assayed using an in-house ELISA [29].