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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

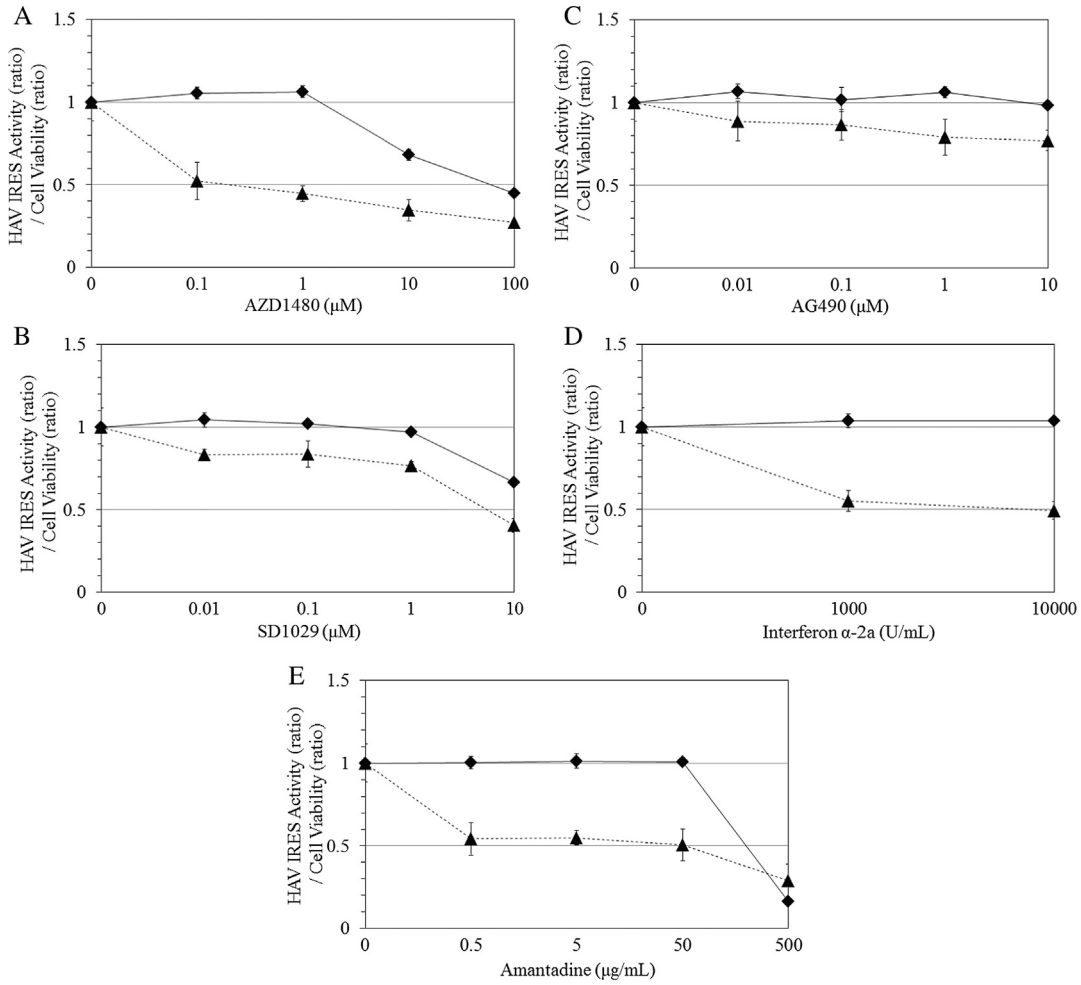

Fig. 1. Effects on cell viability and hepatitis A (HAV) internal ribosomal entry-site (IRES) activity in COS7-HAV-IRES cells. (A) AZD1480, (B) SD1029, (C) AG490, (D) interferon α-2a, (E) amantadine. Cell viability (black diamonds) was evaluated using the MTS assay (Promega). HAV IRES activities (black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

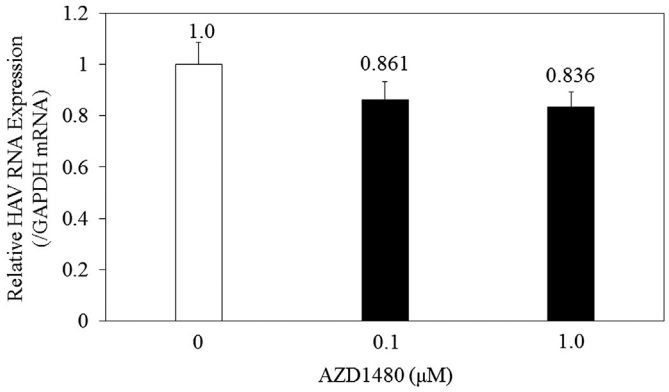

Fig. 2. Inhibition of HAV HA11-1299 genotype IIIA strain replication by AZD1480 in Huh7 cells. Huh7 cells were infected with the HAV HA11-1299 genotype IIIA strain at an MOI of 0.1 24 h after treatment with 0.1 μM or 1 μM AZD1480. At 96 h post-infection, intracellular HAV RNA levels were evaluated by 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

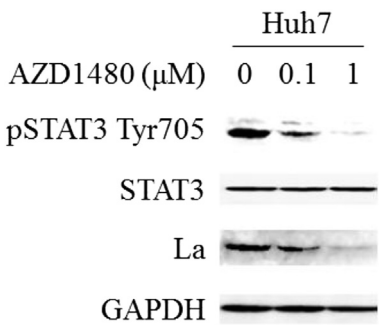

Fig. 3. Effects of AZD1480 on STAT3 and La expression in Huh7 cells. Forty-eight hours after treatment with or without AZD1480, cell lysates were analyzed for phosphorylated-(Tyr-705)-STAT3, STAT3, La and GAPDH expression using specific antibodies.

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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