BRIEF REPORT

Inhibition of hepatitis E virus replication by proteasome inhibitor is nonspecific

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Abstract The ubiquitin proteasome system plays important role in virus infection. A previous study showed that the proteasome inhibitor MG132 could potentially affect hepatitis E virus (HEV) replication. In this study, we found that MG132 could inhibit HEV and hepatitis C virus (HCV) replication-related luciferase activity in subgenomic models. Furthermore, treatment with MG132 in a HEV infectious model resulted in a dramatic reduction in the intracellular level of HEV RNA. Surprisingly, MG132 concurrently inhibited the expression of a luciferase gene used as a control as well as a wide range of host genes. Consistently, the total cellular RNA and protein content was concurrently reduced by MG132 treatment, suggesting a nonspecific antiviral effect.

Abbreviations

UPS	Ubiquitin proteasome system
HEV	Hepatitis E virus
HCV	Hepatitis C virus
RdRp	RNA-dependent RNA polymerase
PGK	Phosphoglycerate kinase
TYMV	Turnip yellow mosaic virus
HAV	Hepatitis A virus
RP2	Human retinitis pigmentosa 2
CyA	Cyclophilin A
CyB	Cyclophilin B
CD81	Cluster of differentiation 81
IMPDH2	Inosine-5′-monophosphate dehydrogenase 2

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Introduction

The ubiquitin proteasome system (UPS), which serves as a major pathway for protein degradation and modification in eukaryotic cells, can be utilized by many types of viruses [1–3]. Previous studies have demonstrated that UPS can regulate viral RNA-dependent RNA polymerase (RdRp), which mediates viral RNA synthesis [1, 4–6]. In addition, UPS can also regulate ubiquitylation and degradation of some viral structural proteins [7–9] and thus represents a potential antiviral target.

Hepatitis E virus (HEV) is a single-strand positive-sense RNA virus that belongs to the family *Hepeviridae*. It is a small non-enveloped virus with a 7.2-kb RNA genome, which is capped at the 5′ termini and polyadenylated at the 3′ termini [10]. Outbreaks of hepatitis E occur periodically throughout the developing world. It typically causes an acute and self-limiting infection, but fulminant hepatitis and high mortality (reaching 25 %) have been described in cases of pregnant women. In the western world, HEV mainly affects immunocompromised patients with a high risk of developing chronic hepatitis [11]. However, no proven medication is available to treat hepatitis E. A recent study reported potent antiviral effects of a well-known proteasome inhibitor, MG132, against HEV [12]. In a *Renilla*-luciferase-coupled HEV replication model, the authors showed that treatment with MG132 resulted in dramatic reduction of HEV-related luciferase activity [12].

These important findings have inspired us to further evaluate the effects of MG132 in two HEV cell culture models.

Materials and methods

In this study, two human hepatoma cell line (Huh7)-based HEV cell culture models were employed: a subgenomic HEV replicon containing *Gaussia* luciferase reporter (p6-Luc) in which the accumulation of secreted luciferase serves as a reporter for HEV replication, and a full-length infectious model (p6) in which Huh7 cells were electroporated with full-length HEV genomic RNA (Kernow-C1 p6 clone, GenBank accession number JQ679013) [13]. Two firefly luciferase cell models were also used: a cell line for normalization in which stable expression of luciferase is driven by a phosphoglycerate kinase (PKG) promoter (Huh7-PGK) and a hepatitis C virus (HCV, also a single-strand positive-sense RNA virus) subgenomic cell culture model (Huh7-ET) [14]. The *Gaussia* luciferase and firefly luciferase activity were measured as described previously [13] using a Lumi Star Optima luminescence counter (BMG Lab Tech, Offenburg, Germany). MTT assays were performed as described previously [15]. The absorbance of each well was read using a microplate absorbance reader (Bio-Rad) at a wavelength of 490 nm.

RNA was isolated using a Macherey-Nagel NucleoSpin RNA II Kit (Bioke, Leiden, The Netherlands) and quantified using a NanoDrop ND-1000 (Thermo, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (Takara Bio Inc.). HEV, GAPDH, RP2 (human retinitis pigmentosa 2), CyA (cyclophilin A), CyB (cyclophilin B), CD81 (cluster of differentiation 81) and IMPDH2 (inosine-5′-monophosphate dehydrogenase 2) were quantified by SYBR-Green-based real-time PCR. The HEV primer sequences were 5′-ATTGGCCAGAAGTTGGTTTTCAC-3′ (sense) and 5′-CCGTGGCTATAATTGTGGTCT-3′ (antisense), the primer sequences for the housekeeping gene GAPDH were 5′-TGTCCCCACCCCCAATGTATC-3′ (sense) and 5′-CTCCGATGCCTGCTTCACTACCTT-3′ (antisense), and the primers for the housekeeping gene RP2 were 5′-CCCATTAAACTCCAAGGCAA-3′ (sense) and 5′-AAGCTGAGGATGCTCAAAGG-3′ (antisense). The primer sequences for CyA were 5′-GGCAAATGCTGGACCC AACACA-3′ (antisense) and 5′-TGCTGGTCTTGCCATTC CTGGA-3′ (sense), and the primers for CyB were 5′-AACGCAGGCAAAGACACCAACG-3′ (antisense) and 5′-TCTGTCTTGGTGCTCTCCACCT-3′ (sense). The primers for CD81 were 5′-CTGCTTTGACCACCTCAGTGCT-3′ (antisense) and 5′-TGGCAGCAATGCCGATGAGGTA-3′ (sense), and the primers for IMPDH2 were 5′-AG-TGGCTCCATCTGCATTACGC-3′ (antisense) and 5′-GGATTCCTCCATCAGCAATGACC-3′ (sense).

For Western blot, 100,000 cells were seeded in a 6-well plate and treated with MG132 for 48 h. Cell lysates (300 μl) were heated for 5 minutes at 95 °C followed by loading 30 μl of sample onto a 10 % sodium dodecyl sulfate polyacrylamide gel and separating by electrophoresis. Mouse β-actin antibody (1:1000) was used as primary antibody. For SDS-PAGE, after electrophoresis for 90 min at 120 V, the gel was stained in Coomassie brilliant blue solution and distained.

Results

Consistent with a previous study [12], treatment with 1 μM and 10 μM MG132 did not significantly impair cellular metabolic activity or viability as determined by MTT assay after 24 h (Fig. 1A) and 48 h (Fig. 1B), although a minor inhibitory effect was observed when cells were treated with
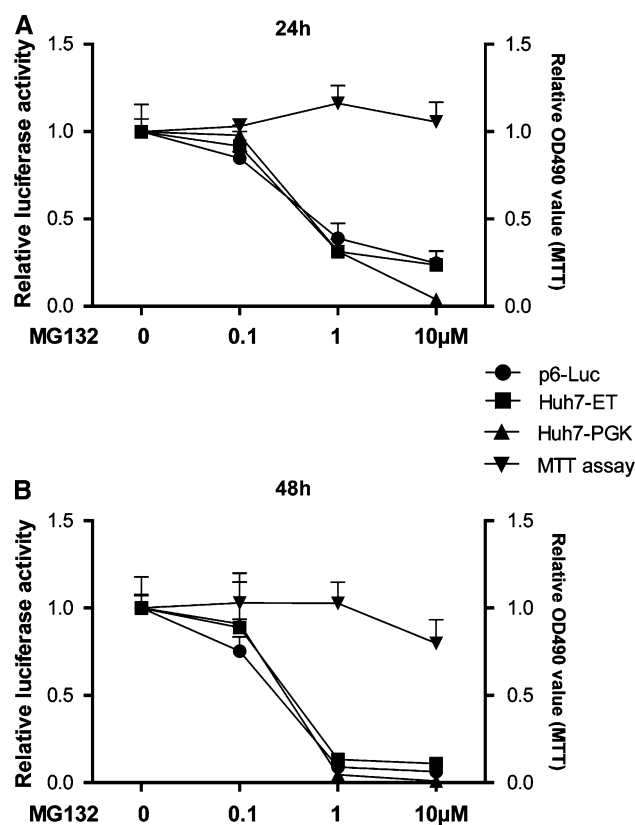

Fig. 1 Nonspecific effects of the proteasome inhibitor MG132 on luciferase activity. Treatment with MG132 after 24 h (A) and 48 h (B) resulted in dramatic reduction of luciferase activity in subgenomic HEV replicon (p6-Luc) (mean ± SD, n = 12), HCV replicon (Huh7-ET) (mean ± SD, n = 4) and Huh7 cells constantly expressing a control luciferase gene under the control of the PGK promoter (Huh7-PGK) (mean ± SD, n = 4). MG132 treatment did not strongly affect cellular metabolic activity or viability, as determined by MTT assay (OD$_{490}$ value) (mean ± SD, n = 4), although a minor inhibitory effect was observed after treatment at 10 μM for 48 h

10 μM MG132 for 48 h. As expected, treatment with 1 μM MG132 potently inhibited HEV-replication-related *Gaussia* luciferase activity in the p6-Luc model (Fig. 1) after 24 h and 48 h. Furthermore, we tested this proteasome inhibitor in the Huh7-based hepatitis C virus subgenomic model (Huh7-ET). Consistently, MG132 inhibited HCV-coupled firefly luciferase activity (Fig. 1). Surprisingly, when Huh7-PGK cells were treated with MG132, the control firefly luciferase activity driven by the PGK promoter was also potently inhibited (Fig. 1). These results raised concerns regarding the specificity of the effect of MG132 on viral replication.

To investigate further, the HEV infectious model (p6) was treated with MG132 for 48 h. The relative levels of HEV viral RNA and two host reference genes (GAPDH and RP2) were quantified by SYBR-based qRT-PCR. As shown in Fig. 2A, treatment with 1 or 10 μM MG132 resulted in a significant decrease in intracellular HEV RNA by 32 ± 19 % and 76 ± 24 % (mean $\pm$ SD, n $= 6$, $p < 0.01$), respectively. Strikingly, the expression levels of two references genes, GAPDH and RP2 were concurrently decreased. In addition, the expression of four other host genes that we tested, CyA, CyB, CD81 and IMPDH2, also decreased simultaneously (Fig. 2B). These results confirm that the effect of MG132 is nonspecific.

Next, we measured the RNA concentration and total protein content of the cells after MG132 treatment and we found that MG132 treatment (1 μM and 10 μM) drastically reduced the total cellular RNA content (Fig. 3A). Furthermore, cells that were treated with MG132 and lysed showed reduced cellular protein expression. As shown in Fig. 3B, the protein level of internal reference β-actin was decreased after treatment with 1 μM and 10 μM MG132, and the total protein content was also reduced (Fig. 3C). However, the effects of MG132 at the protein level were

less profound than that at the RNA level. These results suggest that MG132 inhibits expression and translation of a broad range of genes rather than having a specific effect on viral infection.

Discussion

There is substantial evidence suggesting that the cellular UPS is associated with viral infection. RdRp, the essential enzyme for viral replication, can be regulated by UPS in turnip yellow mosaic virus (TYMV) [1], Sindbis virus [4], hepatitis A virus (HAV) [5] and HCV [6] infections. Virus-encoded proteases cleave viral polyprotein proteolytically but can also mediate the processing of many host proteins [16]. Mature 3C proteases of HAV and encephalomyocarditis virus (EMCV) have been shown to be subject to rapid, ubiquitin-mediated protein degradation [17, 18]. As a combat strategy, some viral proteases have been shown to contain de-ubiquitinating enzyme activity. Papain-like cysteine proteases of SARS coronavirus [19], HEV [20] and foot-and-mouth disease virus (FMDV) [21] have the ability to hydrolyze ubiquitinating substrates. Therefore, modulating the UPS represents as a potential antiviral strategy.

Treatment with the proteasome inhibitor MG132 has been shown to decrease the titer of porcine circovirus type 2 (PCV2) at an early stage of infection [22]. Treatment with MG132 has also been shown to decrease the activity of *Renilla* luciferase expressed from an HEV replicon [12]. However, our study raised concerns regarding the specificity of the effect of MG132 on HEV replication. Although we confirmed the inhibitory effects on luciferase activity in both the HEV and HCV replicon models, MG132 also inhibited constitutively expressed luciferase in control
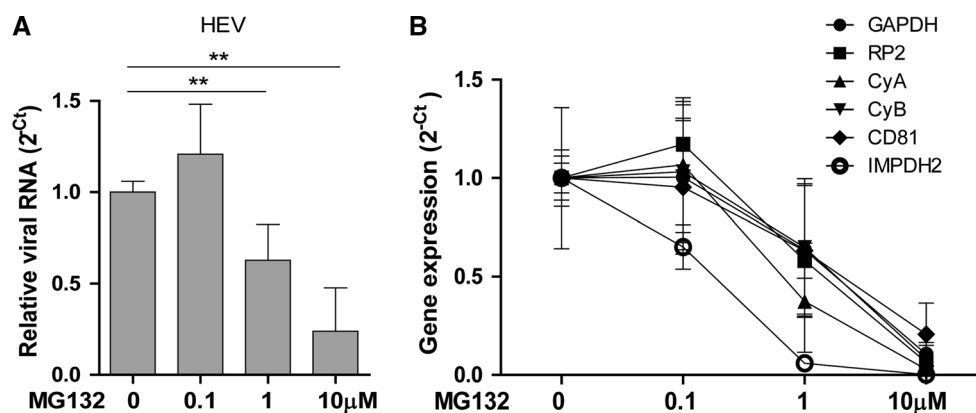

Fig. 2 Significantly decrease in HEV viral RNA levels after MG132 treatment (A). (B) The expression of two reference genes (GAPDH, RP2) and four host genes (CyA, CyB, CD81 and IMPDH2) were concurrently inhibited by treatment with MG132. Relative gene expression was quantified by qRT-PCR. Data are presented as 2^{-Ct} and normalized to the untreated control (mean $\pm$ SD, n $= 6$). **$P < 0.01$
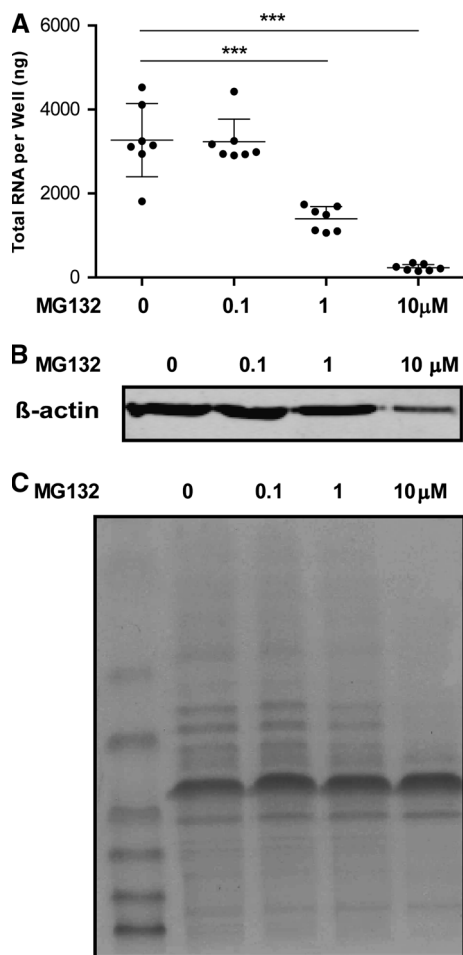
Fig. 3 Decrease in cellular RNA and protein content after MG132 treatment. (A) Total intracellular RNA in cells was dramatically reduced by MG132 treatment after 48 h. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (mean ± SD, n = 7). (B) Protein expression of internal reference β-actin was inhibited by MG132 (in particular with 10 μM) treatment after 48 h. The same volumes of cell lysates were loaded, and the protein level was determined by Western blot. (C) Total intracellular protein was reduced by MG132 treatment (in particular with 10 μM) after 48 h. The same volumes of cell lysate were loaded, and the gel was stained in Coomassie brilliant blue solution and distained. **$P < 0.01$ ***$P < 0.001$

cells. Furthermore, in the full-length HEV model, although MG132 treatment reduced HEV RNA levels, it also simultaneously inhibited the expression of reference genes and other host genes. We further demonstrated that MG132 dramatically decreases the levels of total intracellular RNA and protein, which explains its nonspecific effect on viral infection.

It is not surprising that inhibition of this system could exert variety of effects on cell physiology, since the UPS plays an essential role in the processing of cellular proteins. Proteasomes promptly degrade ubiquitylated proteins [23], and some of these proteins are important mediators of cell-

cycle progression and apoptosis [24]. MG132 has been shown to induce the expression of death receptor 5 (DR5), a receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), resulting in enhanced sensitivity to TRAIL-induced apoptosis in cancer cells [25, 26]. Thus, inhibition of this major intracellular protein degradation pathway could nonspecifically affect viral infection, but we do not fully exclude that UPS may also specifically modulate certain viruses [2].

In summary, this study demonstrated that inhibition of HEV infection by the proteasome inhibitor MG132 is nonspecific. Thus, we should be careful in interpreting data regarding the effects and mechanisms of proteasome inhibitors on viral infection. Although proteasome inhibitors are in the preclinical phase of testing as anticancer agents [24, 27], we would call for caution in developing proteasome-targeted antiviral therapies.

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