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Bardoxolone methyl as a novel potent antiviral agent against hepatitis B and C viruses in human hepatocyte cell culture systems.

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ABSTRACT

Antiviral drugs against hepatitis B virus (HBV) relieve symptoms experienced by patients with hepatitis; however, these drugs cannot eliminate HBV infection from all patients completely. On the other hand, direct antiviral agents (DAAs) against hepatitis C virus (HCV) can achieve near-complete elimination of HCV infection. However, recent reports have claimed that DAAs pose a risk for HBV reactivation among patients with HBV and HCV co-infection. This suggests that an effective anti-viral strategy for both HBV and HCV would be extremely useful. We hypothesized that an activator of nuclear factor-erythroid factor 2 (Nrf2) could be a candidate, because heme oxygenase-1 (HO-1), a product of the Nrf2-target gene, was shown to be related to suppression of genome replication in both HBV and HCV. In this study, the potential of bardoxolone methyl (BARD), an Nrf2 activator, was examined in cell culture systems against HBV and HCV. We investigated that BARD had a suppressive effect on the production of extracellular HBV DNA in several HBV culture systems. In addition, BARD treatment reduced the levels of intracellular HBV pregenome RNA (pgRNA), a transcript from the HBV genome and a template of HBV genome replication. HCV genome replication was also suppressed in HCV subgenomic replicon-bearing cells by BARD treatment. BARD might be a novel treatment for patients with HBV and HCV co-infection.

1. Introduction

Over 250 million people have chronic hepatitis B virus (HBV) infection (Levrero et al., 2018), and HBV is responsible for 887,000 deaths/year, mostly due to HBV-related cirrhosis and HBV-related hepatocellular carcinoma (Schweitzer et al., 2015). Nucleos(t)ide analogue drugs such as lamivudine, adefovir, entecavir (ETV), and tenofovir have been approved for HBV treatment. Nucleos(t)ide analogue drugs show a potent anti-HBV effect through inhibition of reverse transcription of the HBV genome in almost all HBV patients. However, considering their anti-HBV mechanism, these drugs do not impact the covalently closed circular DNA (cccDNA) form of the HBV genome in the hepatocyte nucleus. Therefore, when treatment is stopped, viral replication can potentially resume by using residual cccDNA as the template for transcription of HBV RNAs, and cause relapse of viral hepatitis. Thus, long-term treatment with these drugs is required, which could lead to the emergence of a drug-resistant virus. Although there are direct antiviral agents (DAAs) that are highly effective against hepatitis C virus (HCV) infection, DAAs have a risk for HBV reactivation among patients with HBV and HCV co-infection (Yeh ML et al., 2017). In 2016, the US Food and Drug Administration issued a warning regarding the risk of HBV reactivation in patients receiving DAA therapy for HCV infection. HBV reactivation can occur soon after the start of DAA therapy; thus, monitoring liver enzymes during DAA therapy is

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Abbreviations

HO-1	heme oxygenase 1
Nrf2	nuclear factor-erythroid 2 related factor 2
BARD	bardoxolone methyl
pgRNA	pregenome RNA
HBV	hepatitis B virus
HCV	hepatitis C virus
IFN	interferon
ETV	entecavir
cccDNA	covalently closed circular DNA
DAAs	direct antiviral agents
HBx	HBV protein X
FAS	fatty acid synthase
NTCP	sodium taurocholate transporting polypeptide
TPV	Telaprevir
JFH	Jikei Fulminant Hepatitis

important in patients at risk (Pockros PJ, 2017).

There is no treatment for patients with emergent HBV resistant to nucleos(t)ide analogues. Therefore, novel drugs with different mechanisms for HBV and HCV treatment are needed. We attempted to explore a novel drug that has anti-HBV and anti-HCV effects with an antiviral mechanism different from those of current DAAs and nucleos(t)ide analogues.

It is well known that viruses utilize various host factors for infection and proliferation. Recently, anti-HBV activity of heme oxygenase-1 (HO-1) through repression of HBV replication has been reported (Protzer et al., 2007). Specific porphyrin compounds, including biliverdin, a physiological product of HO-1 enzymatic activity in hepatocytes, were reported to inhibit the priming step of reverse transcription of the HBV genome by interfering with the interaction between HBV reverse transcriptase and HBV pregenomic RNA (pgRNA) (Lin and Hu 2008). With regard to HCV, it has also been reported that lucidone and sulforaphane (SFN) suppress HCV replication through HO-1 induction (Chen WC et al., 2013 and Yu et al., 2016). It is well known that HO-1 gene expression is regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). Therefore, the Nrf2 activator bardoxolone methyl (BARD) was considered a potential anti-HBV and -HCV drug, and an especially useful option for patients with HBV and HCV co-infection. BARD showed an ameliorative effect in diabetic nephropathy patients with type 2 diabetes by suppression of oxidative stress and inflammation in a phase 2 clinical study (Dinkova-Kostova et al., 2005), and its safety in humans was confirmed in a phase 1 clinical study (Pergola et al., 2011). Moreover, a phase 3 clinical study named AYAME was also initiated for diabetes nephropathy. If BARD is approved, it might be easy to make drug reposition from diabetic nephropathy to HBV and HCV treatment. Therefore, in this study, BARD was evaluated as an anti-HBV and anti-HCV reagent in cell culture models of HBV and HCV.

2. Material and methods

2.1. Cell culture

2.1.1. HBV assay

HepG2.2.15.7 cells, which were subcloned from HepG2.2.15 cells and produce a high titer of HBV genotype-D (Sakurai et al., 2017), HepG2-hNTCP-C4 cells, which produce the HBV receptor sodium taurocholate transporting polypeptide (NTCP) (Iwamoto et al., 2014), and Hep38.7-Tet cells, which control the transcription of pgRNA from HBV genotype-D genome by tetracycline treatment (Ogura et al., 2014), were maintained as described previously (Aly et al., 2016). PXB-cells, primary hepatocytes freshly isolated from PXB mouse livers (consisting of > 90% pure human hepatocytes), were purchased from Phoenix Bio Co. (Hiroshima, Japan) and cultured in PXB-cell medium as previously reported (Ishida et al., 2015).

2.1.2. HCV assay

LucNeo#2 and Huh-7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously (Nio et al., 2016).

2.2. Plasmids

HBV gene (genotype Ae, Bj35, C, D, and ETV-resistant (ETVr) coding plasmids (pHBV-Ae, pHBV-Bj35, pHBV-C, pHBV-D, and pHBV-ETVr) were described previously (Sugiyama et al., 2006 and Hayashi et al., 2015).

2.3. Compounds

Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). ETV and BARD (Methyl 2-cyano-3,12-dioxooleana-1,9 (11)-dien-28-oate, CDDO-Me, CDDO methyl ester) were purchased from Funakoshi Co. (Tokyo, Japan). Telaprevir (TPV) and Daclatasvir (DCV) were obtained from Cosmo Bio Co. (Tokyo, Japan).

2.4. Time course and inhibitory concentration 50 (IC_{50}) of BARD for suppression of HBV DNA production

HepG2.2.15.7 cells were seeded on 24-well collagen type-I-coated plates (Corning Inc., Corning, NY, USA) and treated with DMSO containing 0.03, 0.1, and 0.3 μM BARD, and ETV at 3.7 nM, for 24, 48 and 72 h. HepG2.2.15.7 cells were also treated with BARD at 0.1–400 nM for 72 h. The culture medium of the cells was collected and the HBV DNA in the supernatant was quantitated by qPCR. For measurement of cell viability, cells were treated with BARD at 0.5–1.5 μM for 72 h, followed by XTT assay.

2.5. Effect of BARD on various subtypes of HBV and HBV proliferation in PXB-cells

HepG2-hNTCP-C4 cells were seeded into collagen type-I-coated 10 cm dishes (Corning Inc.) and incubated for 24 h. Then, the cells were transfected with pHBV-Ae, pHBV-Bj35, pHBV-C, and pHBV-ETVr plasmids using X-treme GENE HP DNA transfection reagent according to the manufacturer's protocol (Roche Diagnostics, Basel, Switzerland). At 7 days after transfection, cells were passaged to 24-well collagen-coated plates and cultured overnight. The next day, the cells were treated with 0.3 μM BARD and 3.7 nM ETV. After 6 days, the culture medium was collected and HBV DNA in the supernatant was quantitated by qPCR. PXB-cells were seeded on 24-well collagen type-I-coated plates and infected with HBV for 24 h with 0.3 μM BARD and 3.7 nM ETV as described previously (Iwamoto et al., 2014). Cells were washed twice with phosphate buffered saline (PBS, Nacalai Tesque). Then, medium was changed at 24 and 48 h after infection to medium containing 0.3 μM BARD and 3.7 nM ETV. The cells were incubated for an additional 5 days. On day 7 after infection, extracellular HBV DNA in the culture media was measured by qPCR as previously described (Okamura et al., 2016). The viability of PXB-cells was measured by MTS assay.

2.6. Assessment of effect of BARD on production of intracellular HBV DNA,pgRNA, HBsAg and core protein in HepG2.2.15.7 cells and/or Hep38.7-Tet cells

HepG2.2.15.7 cells were treated with 0.3 μM BARD and 3.7 nM ETV

for 72 h. Intracellular HBV DNA, pgRNA and core protein were detected as described previously (Xun et al., 2013). Hep38.7-Tet cells were seeded on 24-well collagen type-I-coated plates and cultured overnight with medium containing tetracycline hydrochloride (Aly et al., 2016). After washing with PBS, cells were cultured in medium containing 0.3 μM BARD or 3.7 nM ETV but not tetracycline hydrochloride for 72 or 96 h. Extracellular and intracellular HBV DNAs, core protein, HBsAg, and HO-1 mRNA were quantified and viability of the cells was measured by LDH assay. The extracellular HBsAg was quantified by HBs Antigen Quantitative ELISA Kit (Beacle, Kyoto, Japan).

2.7. Assessment of contribution of Nrf2 to effect of BARD on extracellular HBV DNA production

Transcription of HBV pregenomic RNA in Hep38.7-Tet cells was induced by shifting to the culture medium without tetracycline to start HBV genome replication as described above. Then, the media containing DMSO, 3.7 nM ETV, or 0.3 μM BARD with various concentrations (0.1–10 μM) of ML385 (Sigma-Aldrich, Missouri, USA), an Nrf2 inhibitor, were used for the medium change. After 4 days' drug treatment, the culture medium was collected and the amount of extracellular HBV DNA was quantified by qPCR and the cell viability was measured by MTS assay using CellTiter kit.

2.8. Assessment of effect of BARD on HCV replication

LucNeo#2 cells were seeded on 24-well collagen type-I-coated plates and treated with BARD at 0.1–10 μM for 72 h. The luciferase activity in LucNeo#2 cells was measured using the Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA) and LUMAT LB 9507 (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturers' protocols. Cell viability was analyzed using XTT assay. The effect of BARD on infectious recombinant HCV derived from cell culture (HCVcc) was also estimated. Huh-7.5 cells were infected with HCVcc generated from Huh7.5 cells transfected with *in vitro* synthesized Jikei Fulminant Hepatitis (JFH) 1^{E2FL} RNA as described previously (Miyanari et al., 2007). Then, the cells were treated with BARD at 0.1 and 0.5 μM for 72 h. HCV RNA was quantified by qRT-PCR. The amounts of HCV RNA were normalized to the amounts of intracellular total RNA. As a positive control, 0.3 μM TPV was used as previously described (Nio et al., 2016).

2.9. Assessment of effect of BARD on production of HCV NS5A protein

LucNeo#2 cells were seeded on collagen type- I -coated 12-well plates (1×10^5 cells/well). After overnight culture, the culture medium was shifted to drug-containing medium. After 3 days' drug treatment, the cellular protein samples were collected by 2x Laemmli sample buffer with 5% β-mercaptoethanol (Nacalai tesque Kyoto, Japan). HCV NS5 protein in total cellular protein was detected by Immunoblot analysis.

2.10. Assessment of contribution of Nrf2 to effect of BARD on HCV genome replication

LucNeo#2 cells were seeded on 48-well plates (1×10^4 cells/well). After overnight culture, the cultue medium was shifted to the medium containing 0.1% DMSO, 3.7 nM DCV, or 0.3 μM BARD with various concentrations (10 and 30 μM) of ML385. After 3 days' drug treatment, the luciferase (Luc) activity in LucNeo#2 cells and the cell viability were measured as described above.

2.11. XTT cell proliferation assay

The cytotoxicity of drug treatment was estimated by XTT (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium) inner salt) assay using a Cell Proliferation Kit II (Roche Diagnostics) for HepG2.2.15.7, HepG2-hNTCP-C4, and LucNeo#2 cells according to the manufacturer's protocols.

2.12. Lactate dehydrogenase (LDH)cytotoxicity assay

The cytotoxicity of the drug treatment on Hep38.7-Tet cells was estimated using the LDH Cytotoxicity Detection Kit (Takara, Otsu, Japan) according to the manufacturer's protocols, with a slight modification of the concentration of positive control, Triton X-100 (Nacalai, Kyoto, Japan). For the positive control, 2% Triton X-100 was used to completely lyse the cells.

2.13. MTS cell proliferation colorimetric assay

MTS assay was conducted for cell toxicity on PXB-cells. Supernatants of PXB-cells were evaluated using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's protocols.

2.14. Nucleic acid extraction

Isolation of total DNA from cells and culture media was performed the using DNeasy Blood and Tissue Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions, except the culture media was pretreated with micrococcal nuclease as described by the manufacturer's protocol (New England Biolabs, Inc., Ipswich, MA, USA). Total RNA from these cells was extracted using the RNeasy Plus Mini Kit (QIAGEN) or NucleoSpin® RNA Plus kit (Macherey-Nagel GmbH & Co. KG, Duen, Germany).

2.15. qPCR, qRT-PCR, and RT-PCR

HBV DNA was quantified by qPCR using the THUNDERBIRD® Probe qPCR Mix (TOYOBO, Osaka, Japan) using a StepOnePlus™ Real Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described (Sugiyama et al., 2006). For HBV pgRNA detection by qRT-PCR, the RT reaction and qPCR were performed using ReverTra Ace® (TOYOBO) and Universal SYBR Select Master Mix (Applied Biosystems), respectively. The primers used were previously described (Yan et al., 2012). RT-PCR was performed using ReverTra Ace® (TOYOBO). For detection of HO-1 mRNA, forward primer 5′-CTTCTTCACCTTCCC CAACA-3′ and reverse primer 5′-AGCTCCTGCAACTCCTCAAA-3′ were used. For detection of glutathione S-transferase A2 (GSTA2) mRNA, forward primer 5′- TTCCCTAACTTGACCCTTCTTTCAG-3′ and reverse primer 5′- GGACTCCATTCTGCCCCGTAT-3′ were used. For detection of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, forward primer 5′-GCCGCATCTTCTTTTGCGTC-3′ and reverse primer 5′- TCGC CCCACTTGATTTTGGA-3′ were used.

2.16. Immunoblot analysis

Immunoblot analysis was performed as described previously (Kushima et al., 2010) using a mouse anti-HBV core (HBc) protein monoclonal antibody (7B2 mAb) (Okuyama-Dobashi et al., 2015) or anti-HCV NS5A mAb (Austral Biologicals, San Ramon, CA (HCM-131-5). Glyceraldehyde phosphate dehydrogenase (GAPDH) protein was used as a protein loading control, and detected using anti-GAPDH mAb (6C5, MAB374) (Merck Millipore, Burlington, MA, USA).

2.17. Statistics

Data are expressed as the mean ± SD. Data involving more than 2 groups was assessed by Williams' test or one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. Differences between 2 groups were assessed using Student's t-test or Aspin–Welch's

t-test. $^{\#}P < 0.025$ for Williams test and $^{*}P < 0.05$ for Tukey multiple comparison test, Student's t-test, and Aspin–Welch's *t*-test were considered significant.

3. Results

3.1. Time course of BARD effect on HBV production in HepG2.2.15.7 cells

HepG2.2.15 cells, which are stably transfected with a complete HBV genotype-D genome, were treated with BARD at 0.03, 0.1, and 0.3 μM, and ETV at 3.7 nM for 24, 48, and 72 h. BARD significantly reduced the level of nuclease-resistant extracellular HBV DNA after 72 h treatment without cytotoxicity (Fig. 1A and B). Next, BARD at 0.1–400 nM suppressed extracellular HBV DNA production in a concentration-dependent manner. The IC_{50} of BARD on extracellular HBV DNA production was calculated to be 102.7 nM (Fig. 1C) and the 50% cytotoxicity concentration (CC_{50}) was also calculated to be 1.07 μM (Fig. 1D).

3.2. BARD suppressed extracellular HBV DNA from various HBV subtypes, ETV-resistant HBV and PXB-cells infected with HBV

To evaluate the anti-HBV effect of BARD for several HBV genotypes, we used HepG2-hNTCP-C4 cells after transfection with a plasmid encoding a 1.24-fold length of the HBV genome derived from HBV A, B, or C. The genome from ETVr HBV, derived from HBV genotype-D, was also examined. BARD significantly reduced extracellular HBV DNA levels for all HBV genotypes examined (Fig. 2A–C). Furthermore, BARD reduced extracellular HBV DNA from cells transfected with the ETVr HBV genome to ~25% of that from DMSO-treated cells, although ETV was not significantly reduced (Fig. 2D). To confirm the anti-HBV effect of BARD on normal human hepatocytes, PXB-cells, which are primary hepatocytes from chimeric mice with humanized livers, were infected with HBV, with concomitant BARD treatment at 0.3 μM. BARD decreased the level of extracellular HBV DNA to ~60% as compared with DMSO without cytotoxicity was not significant (Fig. 2E and F). To evaluate the effect of BARD in a prolonged treatment, HepG2.2.15.7 cells were treated with BARD for 6 days. As the result, 0.3 μM BARD significantly suppressed the production of extracellular HBV DNA without cytotoxicity at day 6 (Supplemental Fig. 1). These results suggest that BARD effectively inhibits HBV proliferation irrespective of HBV genotypes and cell types.

3.3. Working points of BARD on HBV in HepG2.2.15.7 cells

In order to assess which step of the HBV life cycle was affected by BARD, the levels of intracellular HBV DNA, a marker of HBV genome replication, were evaluated in HepG2.2.15.7 cells treated with BARD and ETV. BARD significantly reduced intracellular HBV DNA and ETV

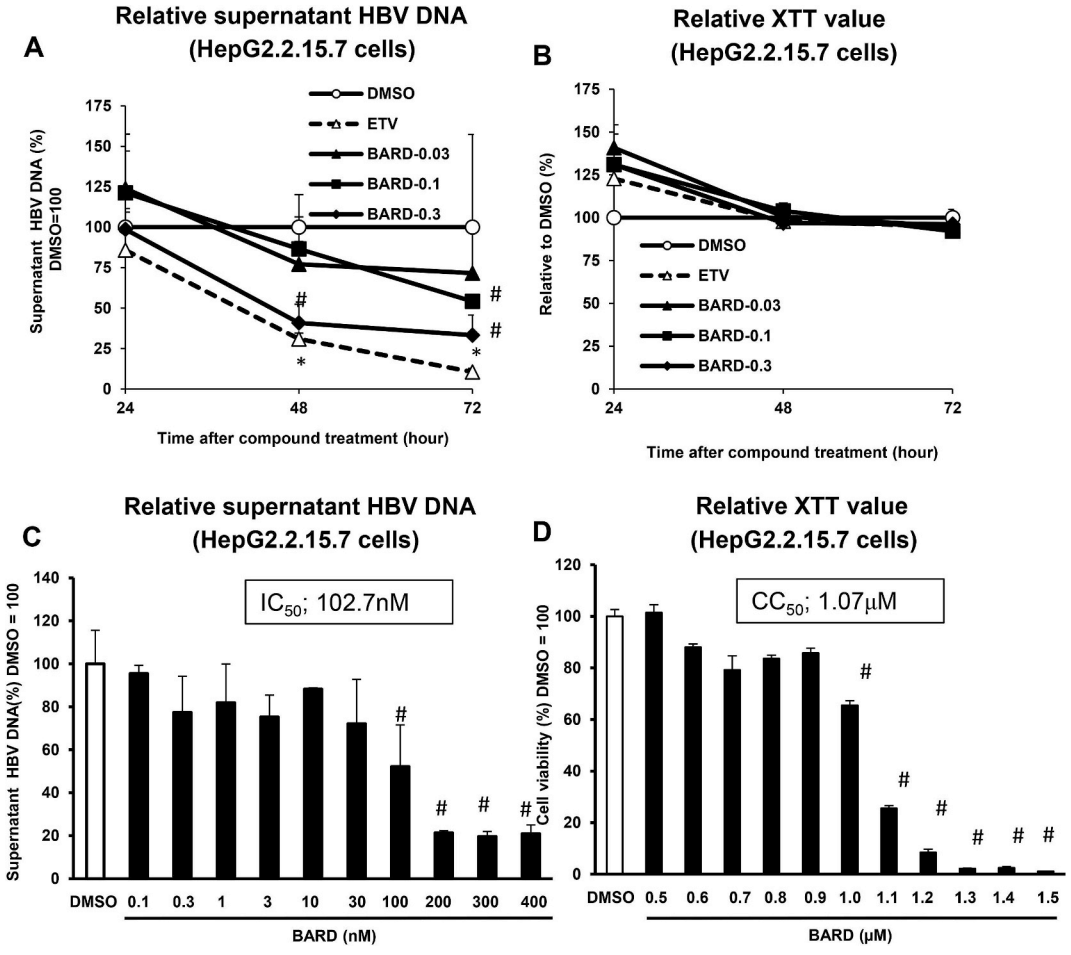

Fig. 1. Time course and inhibitory concentration 50 (IC_{50}) of BARD for suppression of HBV DNA production.
HepG2.2.15.7 cells were treated with BARD at 0.03, 0.1, and 0.3 μM and ETV at 3.7 nM for 24, 48 and 72 h. The extracellular HBV DNA (A) and cell viability (B) were quantitated by qPCR and XTT assay, respectively. HepG2.2.15.7 cells were treated with BARD at 0.1–400 nM for 72 h. Extracellular HBV DNA (C) was quantitated by qPCR. HepG2.2.15.7 cells were treated with BARD at 0.5–1.5 μM for 72 h and cell viability were measured by XTT assay (D). The results are expressed as the mean ± S. D (n = 3 per group). The relative amount of extracellular HBV DNA was normalized to that of DMSO treatment. Data are expressed as the mean ± S. D (n = 3 per group). $^{\#}$, $P < 0.025$ vs. DMSO treatment group by Williams' test. *, $P < 0.05$ vs. DMSO treatment group by Student's t-test.

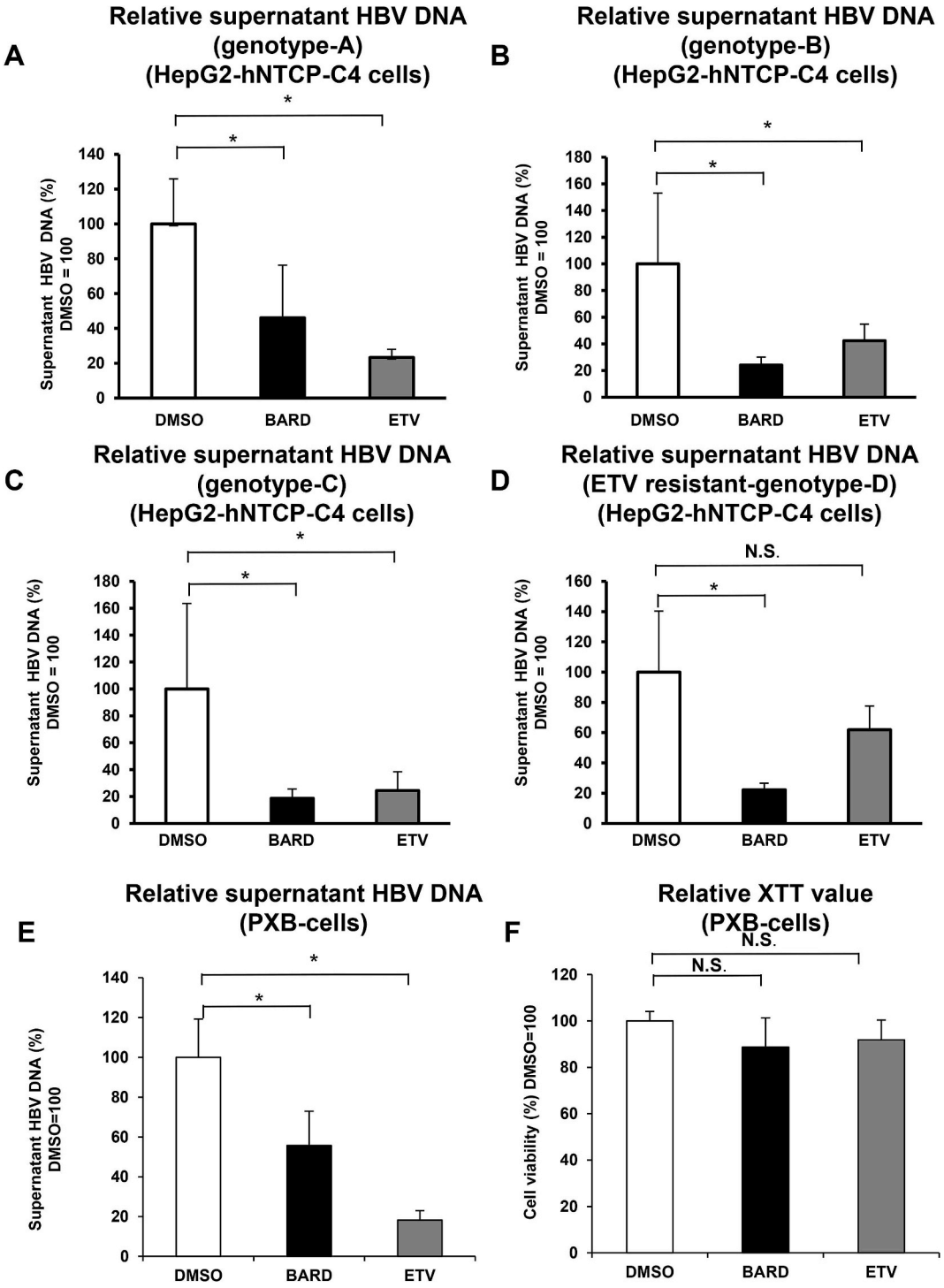

Fig. 2. Effect of BARD on various subtypes of HBV and HBV proliferation in PXB-cells

Seven days after transfection with pHBV-Ae (A), pHBV-Bj35 (B), pHBV-C, (C) and pHBV-ETVr (D) plasmids, HepG2-hNTCP-C4 cells were treated with BARD at 0.3 μM and ETV at 3.7 nM for 6 days. Then, extracellular HBV DNA from each type of plasmid-transfected cells was measured (A–D). PXB-cells were infected with HBV and treated with 0.3 μM BARD and 3.7 nM ETV treatment. 7 days after infection, extracellular HBV DNA levels in the culture media (E) were measured by qPCR. The viability of the cells was measured by MTS assay (F). The results are expressed as the mean ± S. D (n = 3 per group). The relative amount of extracellular HBV DNA was normalized to that of DMSO treatment. *, $P < 0.05$ vs. DMSO treatment group by ANOVA followed by Tukey multiple comparisons test.

was also effective (Fig. 3A). Next, the levels of intracellular HBV pgRNA, one of the transcripts of HBV cccDNA, were evaluated. As shown in Fig. 3B, BARD significantly reduced intracellular HBV pgRNA, while ETV showed only a marginal effect in the same manner as the other nucleoside analogues (Lam et al., 2017). These results suggest that BARD suppresses HBV transcription through the reduction of pgRNA levels in these cells in contrast to ETV. To evaluate the effect of BARD on production of HBV proteins, the levels of HBV core protein in HepG2.2.15.7 cells were examined. HBV core protein level was not significantly changed by BARD treatment, compared with the change of pgRNA (Fig. 3C). It seemed likely that relatively large amount of HBcAg accumulation and relatively high stability of this protein forming capsid

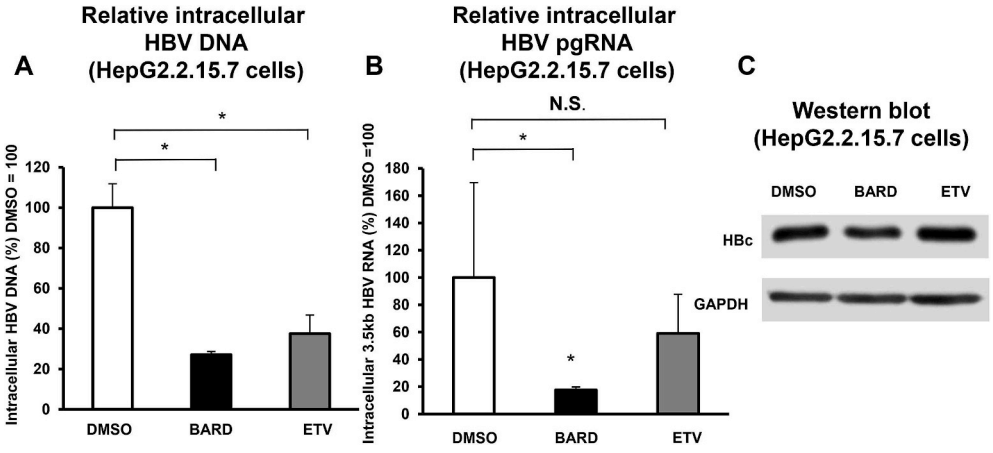

Fig. 3. Targets of BARD effect on HBV in HepG2.2.15.7 cells.
HepG2.2.15.7 cells were treated with BARD at 0.3 μM and ETV at 3.75 nM for 72 h. Intracellular HBV DNA (A) and HBV pgRNA (B) were measured. Intracellular HBV core protein of HepG2.2.15.7 cells (C) after treatment with BARD and ETV was detected. Data are expressed as the mean ± S. D (n = 3 per group). *, $P < 0.05$ vs. DMSO treatment group by ANOVA followed by Tukey multiple comparisons test.

structure in HepG2.2.15.7 cells may be attributed in part to discrepancy between the amount of pgRNA and its translation product in HepG2.2.15.7 cells (Fig. 3C). In HepG2.2.15.7 cells, all HBV proteins are overproduced, thus we postulated that these conditions were not equivalent to those of HBV infected cells. Therefore, in the next experiment, the anti-HBV effect of BARD was analyzed using Hep38.7-Tet cells, which allow for the inducible production of HBV pgRNA and HBV core protein.

3.4. Mechanisms of BARD on HBV

The anti-HBV effect of BARD was examined by using Hep38.7-Tet cells, in which the transcription of HBV genotype-D pgRNA can be controlled by tetracycline (Ogura et al., 2014). BARD significantly reduced extracellular HBV DNA (Fig. 4A), similar to HepG2.2.15.7 cells. Next, the activation of Nrf2 by BARD in this cell line was confirmed by detection of HO-1 gene induction (Fig. 4B). Cell toxicity of BARD was evaluated by LDH assay, and the cell toxicity of BARD treatment was less than 10% of that obtained with 2% Triton X-100 treatment, a positive control of cell toxicity (Fig. 4C). BARD reduced the levels of intracellular HBV DNA (Fig. 4D). BARD also decreased intracellular HBV pgRNA (Fig. 4E) and significantly suppressed HBcAg (Fig. 4F). ETV did not significantly suppress HBcAg and HBV core protein (Fig. 4F). The production of HBV small S antigen (HBsAg) from BARD-treated Hep38.7-Tet cells was also examined by HBs ELISA system. Suppression of HBsAg production was observed in a concentration-dependent manner (Fig. 4G). The suppression of total HBV RNA expression in pHBV/D-IND60-transfected HepG2 cells by the BARD treatment was also observed in northern blot analysis (Supplemental Fig. 2). The reduction of intracellular HBV DNA was also confirmed by Souther blot analysis, although the reduction level did not come up to ETV treatment that completely reduced it in Hep38.7-Tet cells as expected (Supplemental Fig. 3).

To assess the mechanism of reduction of HBV pgRNA by BARD treatment, we performed HBV promoter assay and HBV RNA degradation assay. Compared with DMSO treatment, BARD treatment did not reduce the luciferase activities both in the cells transfected with pHBV core promoter enhancer I + II Luc and pTRE-Tight-Luc. These results suggested that BARD has potential to suppress the transcription from neither HBV core promoter in the HBV genome nor minimal CMV promoter with tetracycline response element (Supplemental Fig. 4). To assess HBV RNA stability by BARD treatment, the time dependent degradation of pgRNA was evaluated in Hep38.7-Tet cells treated with BARD or DMSO after cessation of its transcription by addition of tetracycline. As shown in Supplemental Fig. 5, the amount of pgRNA was more rapidly reduced in the cells treated with BARD than with DMSO, suggesting that BARD would accelerate degradation of HBV pgRNA in the cells. As shown in Supplemental Fig. 6A, the anti-HBV effect of combination treatment of BARD and ETV was also examined by using Hep38.7-Tet cells. The results showed significant reduction of extracellular HBV DNA production from Hep38.7-Tet cells. Calculated combination index (C.I.) values by using CompuSyn software (Nio et al., 2016) showed less than 1 except in the case of extra low dose co-treatment, suggesting a synergistic suppression effect of the overall combination treatment on HBV replication.

To investigate whether the inhibitory effect of BARD on HBV proliferation is dependent on the activation of Nrf2, the effect of, ML385, an Nrf2 inhibitor (Singh et al., 2016), on the suppressed extracellular HBV DNA production from Hep38.7-Tet cells by BARD treatment was examined. As shown in the lower panel of Fig. 4H, the mRNA expression of GSTA2 gene known as an Nrf2 target gene was significantly induced by BARD treatment as expectedly, but that was gradually suppressed by the addition of ML385 in dose dependent manner, indicating that ML385 suppressed the activation of Nrf2. Under these conditions, the suppressive effect of BARD on extracellular HBV DNA production, was reduced by the addition of ML385 in dose dependent manner as well. However, it was observed that ML385 did not fully counteract the effect of BARD even under the treatment with high concentration that completely suppressed the GSTA2 gene induction (Fig. 4H), suggesting that BARD suppressed HBV proliferation not only through activation of Nrf2 signaling pathway but also Nrf2 independent pathway.

3.5. BARD significantly suppressed HCV replication

To investigate the inhibitory effect of BARD on HCV replication, the HCV genotype 1b subgenomic replicon (SGR)-bearing cell line, LucNeo#2 cells, was treated with BARD. BARD significantly suppressed HCV replication in a concentration-dependent manner, without cell toxicity under 1 μM (Fig. 5A and B). The IC_{50} and CC_{50} of BARD on HCV replication were estimated to be 66.8 nM and 2.07 μM, respectively, indicating that BARD potently suppressed HCV replication, and the CC_{50}/IC_{50} ratios were ~30-fold. The effect of BARD on production of HCV viral protein and the amount of SGR RNA in LucNeo#2 cells was also examined by immunoblot analysis for detection of NS5A and RT-qPCR, respectively. As the result, BARD significantly decreased the amount of NS5A as well as that of SGR RNA in LucNeo#2 cells (Fig. 5C). To evaluate the anti-viral effect of BARD in the HCV infection model, Huh-7.5 cells infected with HCV particles were used. HCV particles were produced from Huh7.5 cells transfected with *in vitro* synthetic RNA of Jikei Fulminant Hepatitis (JFH) 1^{E2FL}, which is classified in HCV genotype 2a, as described previously (Miyanari et al., 2007). TPV strongly suppressed HCV RNA, and BARD also significantly suppressed HCV RNA at 0.1 and 0.5 μM in Huh-7.5 cells (Fig. 5D). These results suggested that BARD is possibly effective against HCV irrespective of

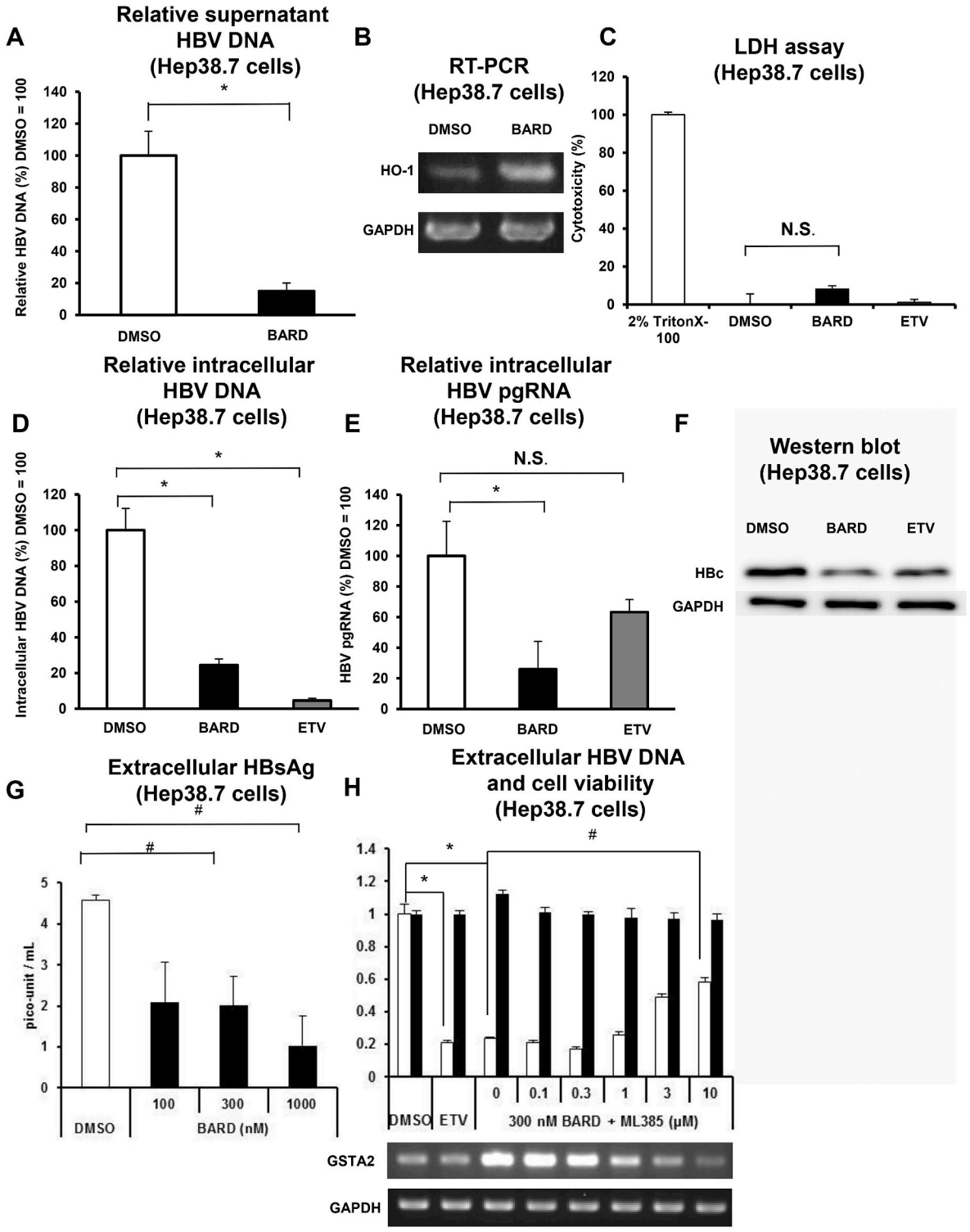

Fig. 4. Targets of BARD effects on HBV in Hep38.7-Tet cells.

Hep38.7-Tet cells were treated with BARD at 0.3 μM for 72 h and extracellular HBV DNA was quantitated by qPCR (A). HO-1 mRNA expression was detected in Hep38.7-Tet cells by RT-PCR after 12 h treatment with BARD at 0.3 μM (B). The viability of Hep38.7-Tet cells, treated with BARD at 0.3 μM and ETV at 3.7 nM for 72 h, were measured by LDH assay (C). Intracellular HBV DNA (D) and HBV pgRNA (E) were and core protein levels (F) of were also analyzed. After 4 days drug treatment, the extracellular HBsAg was quantified by ELISA Kit (G). Hep38.7-Tet cells were co-treated with 300 nM BARD and ML385 at 0.1–10 μM for 4 days. The amount of extracellular HBV DNA in the media (white bar) was evaluated by qPCR and the cell viability (black bar) was measured by CellTiter assay (H). Data are expressed as the mean ± S. D (n = 3 per group). *, $P < 0.05$ vs. DMSO treatment group by the Student's t-test, and #, $P < 0.025$ vs. DMSO treatment group by Williams' test. *$P < 0.05$ vs. DMSO treatment group by ANOVA followed by Tukey multiple comparisons test.

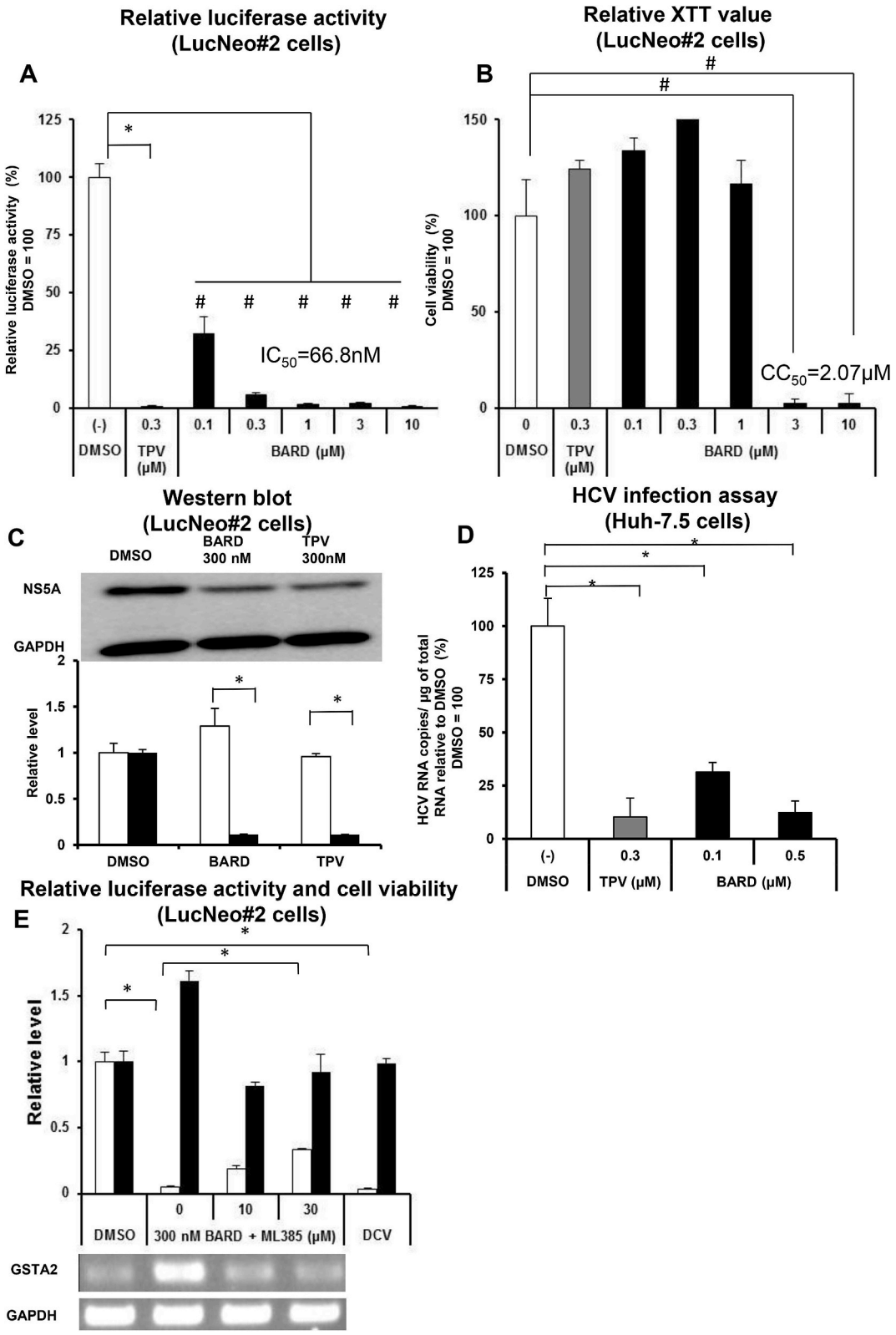

Fig. 5. Effects of BARD on HCV genome replication.

LucNeo#2 cells were treated with BARD at 0.1–10 μM and luciferase activity (A) and XTT absorbance (B) were measured. As a positive control, TPV at 0.3 μM was used. Protein expression levels of HCV NS5A and GAPDH were determined after BARD and TPV treatment for 3 days. Luciferase activity (black bar) and the cell viability (white bar) by CellTiter assay were also measured (C). For infection assay, Huh-7.5 cells, which were infected with HCV, were treated with BARD at 0.1 and 0.5 μM and TPV at 0.3 μM for 72 h. HCV RNA in Huh7.5 cells was quantified (D). LucNeo#2 cells were co-treated with 300 nM BARD and ML385 at 1–30 μM for 3 days. The Luc activity (white bar) was measured by Luc assay and the cell viability (black bar) was measured by CellTiter assay (E). Data are expressed as the mean ± S. D (n = 3 per group). $^{\#}P < 0.025$ vs. DMSO treatment group by Williams test, *, $P < 0.05$ vs. DMSO treatment group by Student's t-test, and $^{*}P < 0.05$ vs. DMSO treatment group by ANOVA followed by Tukey multiple comparisons test.

different genotypes because the genetic distance between HCV1b and HCV2a was evaluated to be huge (Simmonds, 2004). Next, IC_{50} and CC_{50} values of BARD and TPV on HCV replication in JFH1E2FL-infected Huh-7.5 cells was also examined as above. As the result, BARD and TPV inhibited the HCV replication with an IC_{50} value of 61.8 nM and 3.02 nM, respectively (Supplemental Figs. 7A and C). The CC_{50} values of BARD and TPV were also evaluated as 1.41 μM and 71.6 μM, respectively, (Supplemental Figs. 7B and C).

To investigate whether the inhibitory effect of BARD on HCV genome replication is dependent on the activation of Nrf2, the effect of ML385 on the luciferase activity suppressed by BARD in LucNeo#2 cells was examined as described above. As shown in (Fig. 5E), the luciferase activity significantly suppressed by sole treatment with BARD (0 μM ML385), but that was gradually recovered by the addition of ML385 in a dose dependent manner. It, however, seemed likely that the suppressive effect of BARD on HCV genome replication is only partially dependent on Nrf2 activation, because the maximum level of the recovery of luciferase activity by the addition of 30 μM ML385 was only 40% of luciferase activity in the cells without BARD treatment despite no induction of GSTA2 gene under this condition.

4. Discussion

In this study, BARD was first found to be an effective compound against HBV and HCV in cell culture systems. It also should be noted that BARD suppressed the proliferation of various genotypes of HBV, as well as ETVr HBV. With respect to HCV, the Nrf2 activator, SFN, was reported to inhibit HCV replication by suppression of HCV NS3 protease activity and induction of the IFN system through induction of HO-1 gene expression (Yu et al., 2016). A product of HO-1 enzymatic reaction, biliverdin, was reported to have a potential to inhibit HBV genome replication via blocking the interaction between pgRNA and HBV reverse transcriptase (Lin L et al., 2008). As shown in Figs. 4H and 5E, however, the ML385 co-treatment with BARD did not completely rescue the expression of extracellular HBV and Luc activity which were reduced by BARD in Hep38.7-Tet cells and LucNeo#2 cells, respectively. Those results suggest that BARD inhibited the replication of both HBV and HCV in both Nrf2 activation-dependent and -independent manners. As for the Nrf2 activation-dependent pathway, it seemed possible that the induction of IFN-stimulated genes, caused by biliverdin production via HO-1 induction, reported as in the case of HCV (Yu et al., 2016), affected the viral genome replication. It also was observed that BARD treatment significantly decreased the amount of intracellular HBV pgRNA that was reported as an anti-HBV effect of IFNα (Belloni et al., 2012). However, in this study, BARD did not induce the expression of several IFN-related genes, such as interferon-stimulated gene (ISG)15, ISG56 and retinoic acid-indicible gene-I (RIG-I) genes in not only HepG2.2.15.7 and Hep38.7-Tet cells, but also LucNeo#2 cells. (Supplemental Fig. 8). Therefore, the inhibitory effect of BARD on HBV and HCV genome replications did not seem to be due to IFN-pathways. To assess the mechanism of reduction of HBV pgRNA by BARD treatment, BARD did not affect HBV core promoter activity but enhanced HBV pgRNA degradation (Supplemental Figs. 4 and 5). Therefore, reduction of HBV RNA by BARD treatment was likely to be due to an acceleration of HBV RNA degradation, at least in part. However, the detail molecular mechanism of anti-HBV effect of BARD was still unknown at this moment, the host factors and pathways that contribute to anti-HBV and anti-HCV effects of BARD in Nrf2 activation dependent and independent manners should be elucidated in the future study.

From the clinical point of view, BARD showed an ameliorative effect on patients with chronic kidney disease correlated with type 2 diabetes, and a clinical study of BARD with a daily dosage regime (150 mg/day) on these patients is ongoing (Pergola et al., 2011). For cancer thearapy, Gee et al. reported that BARD suppresses HBV large surface protein variant W4P-related carcinogenesis and hepatocellular carcinoma cell proliferation via the inhibition of signal transducer and activator of transcription 3 signaling (Gee et al., 2018). According to our study and their results, BARD might be effective for not only HBV proliferation and but also hepatocellular carcinoma induced by HBV infection. Although there is very narrow selectivity between IC_{50} and CC_{50} on BARD for HBV treatment in Fig. 1, the safety of BARD has been already confirmed in clinical level to some extent. Therefore, safety margin ratio between risk and benefit of BARD on antiviral treatment should be focused in vivo study. In a phase 1 first-in-human trial, in patients with advanced solid tumors and lymphomas, treatment with BARD at an orally administrated dosage of up to 900 mg/day was reported to be well-tolerated. According to the pharmacokinetics data in humans, the Cmax values for 150 and 300 mg/day of oral administration were 16.6 ng/ml (32.8 nM) and 22.7 ng/ml (44.8 nM), respectively (Hong DS et al., 2012). In this study, IC_{50} values for BARD on HBV and HCV were calculated to be 102.7 nM and 66.8 nM, respectively (Figs. 1A and 5A). Therefore, well-tolerated treatment with BARD at an orally administrated dosage of up to 900 mg/day might be over these IC_{50} values for BARD on HBV and HCV in plasma or liver. In this study, we have shown BARD to be an effective to various HBV genotypes, ETV-resistant HBV and HCV in liver cells. In the future study, the precise molecular mechanism of interaction between BARD and HBV lifecycles will provide an important insight into the host cell pathway for suppression of HBV proliferation. According to our results, we suggest that BARD may be an effective treatment option for patients with HBV and HCV co-infection (Norouzian et al., 2016).

5. Conclusions

BARD might be a novel treatment for patients with HBV and HCV co-infection.

Glossary

Telaprevir, an oral HCV non-structural viral protein 3 protease inhibitor.

Entecavir, a guanine analogue that inhibits HBV replication process.

Conflicts of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104537.
The sequence of target-specific primer sets.

ISG15	F	5′ –GCGCAGATCACCCAGAAGAT- 3′
	R	5′ –GTTCGTCGCATTTGTCCACC- 3′
ISG56	as previously described (Tsugawa Y. et al., PLoS One. 2014)	
RIG-I	as previously described (Tsugawa Y. et al., PLoS One. 2014)	
IFN-α	as previously described (Tsugawa Y. et al., PLoS One. 2014)	

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