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Benzimidazole derivative, BM601, a novel inhibitor of hepatitis B virus and HBsAg secretion

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ABSTRACT

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family. HBsAg, greatly outnumbered mature virion, has been mysterious since the discovery of HBV. A novel benzimidazole derivative, BM601, is identified inhibiting the secretion of HBV virions and HBsAg, with 50% effective concentration of 0.6 μM and 1.5 μM, as well as 50% cytotoxicity concentration of 24.5 μM. It has no effect on transcription, protein production, nucleocapsid formation or intracellular HBV DNA synthesis. Immunofluorescence analysis suggests that BM601 might inhibit virion and HBsAg secretion by interfering surface protein aggregation in *trans* Golgi apparatus. Furthermore, BM601 does not trigger cellular stress response or affect HBeAg or host protein secretion. We hypothesize that BM601 is a secretion inhibitor functioning at the level of virion and HBsAg secretion pathway.

1. Introduction

Hepatitis B Virus (HBV) is a partially double-stranded genomic DNA virus belonging to the family *Hepadnaviridae*. It is estimated that 350–400 million people worldwide are chronically infected with HBV (Lavanchy, 2005). HBV has a distinct mode of replication depending on the procedure of covalently closed circular DNA (cccDNA) which transcripts its genome in the host cell nucleus (Nassal, 1999). Upon envelope-mediated attachment to the hepatocytes, partially double-stranded viral genomic DNA (rcDNA) in the nucleocapsid is transported into the nucleus and converted into cccDNA, which serves as the template for the transcription of viral RNAs including pregenomic RNA (pgRNA). Then core proteins encapsidate both pgRNA and polymerase into an icosahedral capsid, also referred to as nucleocapsid, in which viral replication occurred. The majority of the nucleocapsids associated with the envelope proteins for virion maturation and secretion, while the rest are sent back to the nucleus and converted to cccDNA.

The envelope proteins include Large (L), Middle (M) and Small (S) surface protein, sharing the common C-terminal S region. They are expressed from one open reading frame but differ in length because of three in-frame AUG start codons. Accordingly, the three surface proteins encompass preS1/preS2/S, preS2/S and S domain. HBV surface proteins are integrated into the endoplasmic reticulum (ER) membrane, initiated by an N-terminal signal sequence (Eble et al., 1987, 1990; Schmitt et al., 2004). They not only constitute the basic structure of the virion, but also form spherical and filamentous subviral particles (SVP). Assembled in the ER or post-ER, pre-Golgi apparatus (Huovila et al., 1992), SVPs contain mostly S envelope proteins (Chisari et al., 1986). L protein is essential in viral morphogenesis by interacting with the nucleocapsid (Bruss, 1997, 2004) but not necessary for SVPs formation (Lunsdorf et al., 2011). Due to their lack of nucleocapsids, the SVPs are non-infectious. SVPs are greatly involved in the progression of disease especially in immune responses against HBV. It is generally

Abbreviations: 3TC, lamivudine; DMSO, dimethyl sulfoxide; CC_{50}, concentration of 50% cytotoxicity; cccDNA, covalently closed circular DNA; ERGIC, ER-Golgi intermediate compartment; ESCRT, endosomal sorting complex required for transport; HCC, hepatocellular carcinoma; HbeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; EC_{50}, 50% effective concentration; pgRNA, pregenomic RNA; rcDNA, viral genomic DNA; ROS, cellular reactive oxygen species; SEAP, secreted alkaline phosphatase.

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accepted that SVPs, the amount of which is at least 10,000 times of that of virions in the blood of infected individuals (Bruns et al., 1998; Ganem and Prince, 2004), behave as decoys for the immune system, weaken the immune responses toward HBV. HBsAg also down-regulate the innate immune responses. HBsAg suppressed lipopolysaccharide (LPS) and IL-2-induced cytokine production (Vanlandschoot et al., 2002), inhibited DC functions and interfered with LPS-induced activation of ERK-1/2 and c-Jun N-terminal kinase-1/2 in monocytes (Op den Brouw et al., 2009). Noteworthy, the progression of cirrhosis and hepatocellular carcinoma was greatly due to sustained HBsAg secretion (Chisari et al., 1989; Wang et al., 2004; Yang et al., 2009; Wu et al., 2014). Taken together, these findings suggest that HBsAg plays an important role in the progression of chronic hepatitis.

All the studies above spurred our interest into HBsAg inhibitor screening. Using ELISA to detect HBsAg quantitatively, we undertook a high-throughput screening of our molecular library. A series of benzimidazole derivatives was accidently found and confirmed to reduce the secretion of hepatitis B virions and HBsAg. It is the first time that benzimidazole derivatives are reported as virion and HBsAg secretion inhibitors. No toxicity was observed at effective concentrations, and the secretion of host cellular proteins was not affected. We proposed that BM601 might be used as a tool drug to investigate the secretion pathway of HBV and throw lights on anti-HBV studies of benzimidazole derivatives.

2. Materials and methods

2.1. Plasmid

The original plasmid pHBV1.3, containing a 1.3-mer over-length copy of the HBV genotype A2 genome (subtype adw2; GenBank accession number: X02763.1) (Wu et al., 2012) was used to construct the plasmid described below. The pTRE1.3 plasmid is identical to pHBV1.3 with the exception that the HBV DNA genes were inserted into pTRE-Tight Vector in a MCS immediately downstream of the Tet-responsive P_{tight} promoter and respond to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems. Consequently, the P_{tight} is silent in the absence of tetracycline, which means that RNAs transcribed for core protein, polymerase and pgRNA are silent. However, possessing their own promoter, surface proteins are continuously expressed. The pHA-HBs plasmid which contains the HBV S envelope gene with a C-terminally tagged influenza virus hemagglutinin (HA) epitope was a generous present from Dr. Mengji Lu (Institute of Virology, University Hospital Essen, Germany).

2.2. Cell culture and transient transfection

HepG2.2.15 cell line is a HepG2 hepatoma-derived cell line that stably replicates HBV (ayw serotype, GenBank accession number: U95551). It was maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 380 μg/ml G418 (GibcoBRL) (Sells et al., 1987). Human hepatoma cell line Huh7 was grown in DMEM supplemented with 10% FBS. Huh7 or HepG2 cells were seeded in six-well plates at 1×10^6 cells per well and transfected with 1 μg plasmid DNA by Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol. All cell lines were cultured at 37 °C with 5% CO2.

2.3. Cytotoxicity assay

HepG2.2.15 cells were cultured in 96-well plates at 4×10^3 cells per well for 8 days at standard condition. At day 4, culture medium was replaced by newly diluted compound medium. At the 8th day of culture, the supernatant was aspirated for real time PCR assay, and the remaining cell were treated with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] (Tong et al., 2010). Briefly, MTT (final concentration 2.5 mg/ml) reagent was added for 3 h at 37 °C. Then the cells were lysed with MTT Lysis Buffer (10% sodium dodecyl sulfate (SDS) and 50% N,N-dimethylformamide, pH 7.2) at 37 °C for 6 h. Next, the absorbance values (OD) of the lysates were determined using a SpectraMax® 190 absorbance microplate reader at 570 nm.

2.4. Analysis of secreted HBsAg, HBeAg, alpha-fetoprotein (AFP) and alpha-1antitrypsin (AAT)

HBsAg and HBeAg in the cell culture medium were measured using ELISA kits (Sino-American biotechnology company, Henan, China) according to the manufacturer's recommendations. AFP and AAT were also measured using ELISA kits (Mlbio, Shanghai China) according to the manufacturer's recommendations. The medium was diluted to fit the linearity range of ELISA kit.

2.5. Secreted alkaline phosphatase (SEAP) assay

Huh7 cells were transfected with pSELECT-zeo-SEAP plasmid (InvivoGen), and treated with BM601 for 3 days. Quantification of SEAP in cell culture supernatant was performed using SEAP Report Gene Assay Chemoluminescent (Roche) according to the manufacturer's instruction.

2.6. Real-Time PCR assay

HepG2.2.15 cells were cultured in 96-well plates for 8 days as described before. The Blood & Tissue Kit (Qiagen) was used for the extraction of HBV DNA from cell culture supernatants. The progeny DNA was quantified by real-time PCR as described previously (Wang et al., 2009).

2.7. Southern blot analysis

HepG2.2.15 cells were cultured in 6-well plates and treated with BM601 as described above. The cells were collected and lysed with cold lysis buffer (0.5% NP-40, 50 mM Tris–HCl, 1 mM EDTA·2Na, pH 7.0). The cell lysate was digested with 100 U/ml Cryonase cold-active nuclease (TaKaRa) for 30 min at 37 °C, and then incubated with proteinase K at 56 °C for 2 h to breakdown nucleocapsids and release associated HBV DNA. Capsid-associated DNA was purified by Phenol/chloroform extraction and then precipitated in ethanol. Nucleic acid pellets were resuspended in TE buffer and separated in a 0.8% agarose gel followed by Southern blotting in which a DIG-labeled HBV-DNA probe encompassing genome position 463–1499 was used (Yang et al., 2014).

2.8. Northern blot analysis

HepG2.2.15 cells were cultured as described above. Total RNA was extracted from HepG2.2.15 cells by Trizol reagent (Invitrogen). The HBV RNA samples were electrophoresed in 1% formaldehyde agarose gels and transferred to Hybond-N+membrane (Amersham Biosciences) following the protocol of NorthernMax® kit (Ambion). The hybridization was performed with the probe described above for 16 h at 50 °C.

2.9. Immunoprecipitation

HepG2.2.15 and plasmid-transfected Huh7 cells were incubated at 4 °C for 15 min with cold lysis buffer (0.25% NP-40, 50 mM

Tris–HCl, 1 mM EDTA·2Na). Detergent-insoluble materials were removed by centrifugation at 4000 rpm for 10 min at 4 °C. CoIP buffer (50 mM Tris–HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% (w/v) Triton X-100, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl Fuoride (PMSF), 1× protease inhibitor cocktail) were added to 1 ml final volume. The whole-cell lysate was incubated at 4 °C overnight with horse anti-HBsAg polyclonal antibody (Abcam). Then protein A/G agarose beads (beyotime) were added and incubated at 4 °C for 2 h. The immunoprecipitated protein was prepared by boiling in SDS buffer for western blotting.

2.10. Western blot analysis

The samples were boiled in SDS sample buffer for 5 min, applied on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), blotted with primary antibodies, detected secondary antibody by chemiluminescence, and exposed to Kodak X-ray film (Kodak).

2.11. Immunofluorescence analysis

HepG2.2.15 cells were grown on coated glass cover slips. After a 3-day treatment with BM601, cells were fixed and permeabilized with 4% methanol for 10 min at room temperature, and then blocked with 10% BSA in PBS for 1 h. For staining, the cells were then incubated sequentially with primary and secondary antibodies, as follows. HBsAg were stained with horse anti-HBsAg polyclonal antibody (1:50 dilution in 10% BSA, 0.3% saponin, Abcam). An endoplasmic reticulum membrane marker, calnexin, was stained with mouse anti-Calnexin antibody (1:100). A Golgi marker, Giantin, was stained with rabbit anti-Giantin antibody (1:100). (FITC)-conjugated anti-horse immunoglobulin G purchased from Abcam, rhodamine-red-conjugated anti-mouse immunoglobulin G and rhodamine-red-conjugated anti-rabbit immunoglobulin G (1:200 dilution in 10% BSA, 0.3% saponin, invitrogen) were used as secondary antibodies. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was used to stain the cell nucleus at the concentration of 10 μg/ml. Staining were visualized with fluorescence microscope (Leica DMRBE).

2.12. Flow cytometric assay of dichlorofluorescin (DCFH) oxidation

After treatment with BM601 for 3 days, HepG2.2.15 cells were washed by phosphate-buffered saline and incubated in the presence of 50 μM cell permeable fluorescent and chemiluminescent probes, 2′-7′-Dichlorodihydrofluoresceindiacetate (DCFH-DA) (Beyotime), in the dark at 37 °C for 4 h. ROS inducer Rosup (provided by the detection kit) was added at the last 30 min of incubation as positive control to get a final concentration of 250 μg/ml. The green fluorescence of DCF was associated with ROS production and analyzed using FACSCalibur system with CellQuest software (Becton Dickin-son), with a total of 10,000 events collected for each histogram.

2.13. Statistical analysis

Calculations of potency and toxicity were based on data from at least three independent evaluations. The 50% effective concentration (EC_{50}) values were calculated by linear regression analysis and the concentration of 50% cytotoxicity (CC_{50}) were calculated by nonlinear regression analysis using Graphpad Prism 5. The bars indicate standard error of the mean. The Selectivity index (SI) was calculated as EC_{50} /CC_{50}.

3. Results

3.1. BM601 inhibited the secretion of HBV virions and HBsAg particles in vitro

BM601 is a non-nucleoside anti-HBV compound, which delivers anti-HBV activity in HepG2.2.15 cell line. The chemical structure is shown in Fig. 1A. To determine antiviral activity of BM601, HepG2.2.15 cell was treated with gradient concentrations of BM601 for 8 days. Lamivudine (3TC) was used as positive control. As shown in Fig. 1C, extracellular HBV DNA dropped dramatically after the treatment with indicated dose of BM601. Moreover, BM601 significantly inhibited the secretion of HBsAg (Fig. 1D). The 50% effective concentration (EC_{50}) of BM601 for viral release was 0.6 μM and for HBsAg is 1.5 μM. Cytotoxicity was detected under the same conditions as activity determination. After 8 days treatment with BM601, the viability of HepG2.2.15 apparently decreased in a dose-dependent manner, but at concentration lower than 12.5 μM, no cytotoxicity was observed. The CC_{50} of BM601 was 24.5 μM (Fig. 1B), yielding $SI_{HBV\ DNA}$ = 41 and SI_{HBsAg} = 16.3. The toxicity of BM601 was also confirmed by LDH release detection (CC_{50} = 27.9 μM) and WST-8 cell counting kit (CC_{50} = 26.5 μM).

3.2. BM601 had no effect on HBV RNA transcription, DNA replication or nucleocapsid formation

Northern, Southern, and Western blotting analyses were used to measure intracellular HBV RNA, DNA, core protein and nucleocapsid levels after treatment with BM601.To confirm whether BM601 influenced the transcription, RT PCR and Northern blot were used to detect intracellular HBV RNA. HBV had four transcripts and all of them share a common polyadenylation signal. In the RT-PCR experiments, sequence 1928–2400 (represented 3.5 kb RNA), sequence 267–563 (represented 3.5 kb, 2.1 kb and 2.4 kb RNA) and sequence 1414–1744 (represented 3.5 kb, 2.4 kb, 2.1 kb and 0.7 kb RNA) were amplified with specific primers. Both BM601 and 3TC had no effect on the amount of the three PCR products (Fig. 2A), suggesting the inhibiting effect of BM601 was not due to down-regulation of viral RNAs. It was also confirmed by Northern blot that BM601 had no effect on 3.5 kb pregenomic RNA and 2.4/2.1 kb subgenomic RNAs as same as polymerase inhibitor 3TC (Fig. 2B). To determine whether the supernatant virion DNA diminishment was due to the impact of BM601 on HBV DNA replication, Southern blotting was used to detect HBV DNA. None of the SS, DSL or RC forms of HBV DNA changed after treatment with BM601, while 3TC, the positive control, inhibited HBV DNA synthesis effectively (Fig. 2C). HBV core protein expression and nucleocapsid formation were also unaffected by BM601 or 3TC in HepG2.2.15 cells (Fig. 2D). These results above indicated that BM601 might block virion secretion.

3.3. BM601 blocked S surface protein secretion but not production

Subviral particles are mainly comprised with S surface protein. To address whether BM601 inhibited the S surface protein production or blocked the protein secretion, we used a HA-tagged S surface protein expression plasmid (pHA-HBs). After transfection with pHA-HBs, Huh 7 cells were treated with BM601 for 3 days. S surface protein was detected by ELISA and Western blot. As shown in Fig. 3A, BM601 reduced the supernatant surface protein compared to the untreated and 3TC treated samples, but did not interfere with the intracellular surface protein expression. Both the results of ELISA and Western blot showed that BM601 reduced the S protein secretion. Under CMV promoter, the S surface protein was largely expressed. That's the reason why the activity of BM601

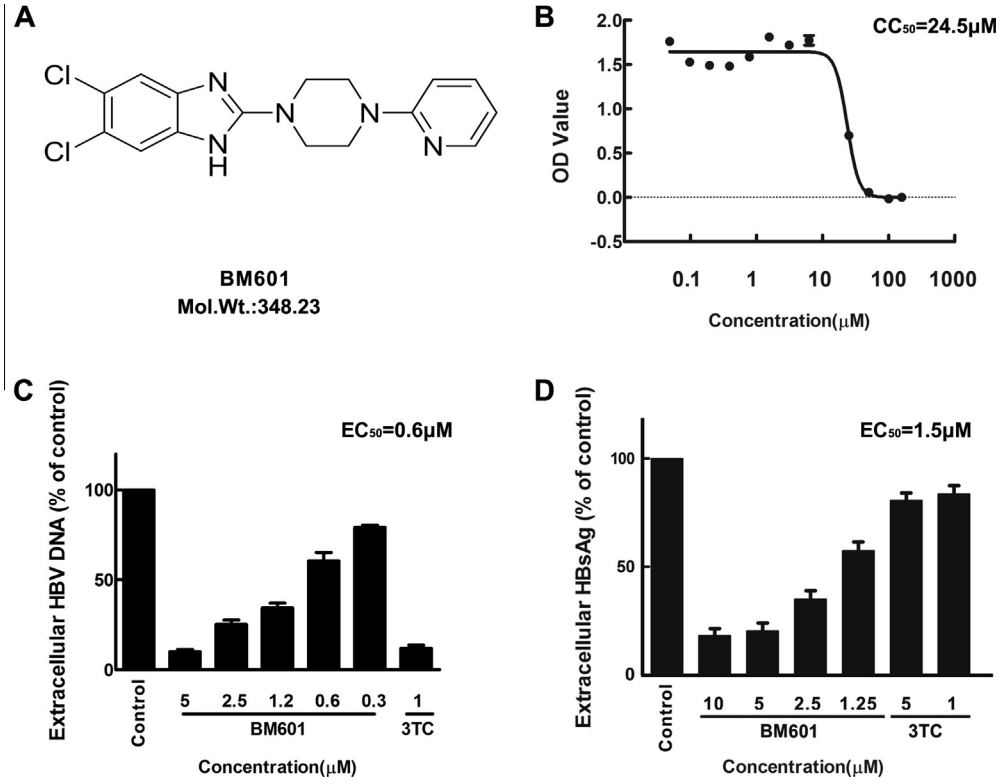

Fig. 1. BM601 inhibited HBV DNA and HBsAg secretion in HepG2.2.15 cell line. (A) Chemical structures of BM601 (a.Mol.Wt. = 348.23). (B) Cytotoxicity of BM601 on HepG2.2.15 cell line using MTT conversion assays, the CC_{50} of BM601 was 24.5 μM. (C) Extracellular HBV DNA quantified using Real time PCR was decreased dose-dependently after treatment with indicated dose of BM601. The EC_{50} of BM601 is 0.6 μM. (D) Extracellular HBsAg of HepG2.2.15 after BM601 treatment was quantified using ELISA. BM601 reduced extracellular HBsAg quantity, with an EC_{50} of 1.5 μM. All data points are the means for triplicate samples, and error bars represent the standard deviations from the mean value. Results shown are typical experiment of at least three individual experiments.

was not as intense as that in HepG2.2.15 cells. The result indicated that BM601 did not act on S surface protein production but on surface protein secretion.

Some previous studies reported that there was a balance among the L, M and S surface proteins. L protein inhibited secretion of subviral particles in a dose-dependent manner, whereas a too-high or too-low L/S protein ratio inhibited virion secretion (Garcia et al., 2009). Production of the three kinds of surface proteins was detected by Western blot using a tet-on system pTRE1.3, producing only surface proteins in the absence of tetracycline. Huh7 cells transfected with pTRE1.3 were treated with either BM601 or 3TC for 3 days, the supernatant HBsAg was detected by ELISA and the cell lysate was analyzed on 12% SDS–PAGE gel as described before. Although secreted HBsAg reduced dramatically, all three kinds of surface proteins had little change after BM601 treatment (Fig. 3B). Therefore, the extracellular surface protein reduction was not related to the intracellular L/S protein ratio.

3.4. BM601 did not affect the interaction between surface protein and core protein

The specific interactions of the viral surface protein with preformed cytosolic nucleocapsids are crucial during HBV envelopment (Bruss, 2004). Once core proteins are assembled into nucleocapsids, they would interact with the envelope proteins of HBV in the endoplasmic reticulum (ER) membranes to form mature enveloped virus particles. Remarkably, the inhibition of surface protein–core protein interaction will decrease the virion secretion dramatically (Asif-Ullah et al., 2006). We therefore tested whether BM601 interfered with the HBV envelopment process by disturbing surface/protein contacts using co-immunoprecipitation system. Huh7 cells transfected with pHBV1.3 or cotransfected with pHA-HBs and pHBc as well as HepG2.2.15 cells were treated with BM601 at concentration of 5 μM or vehicle for three days. Cell lysates prepared with a mild detergent were subjected to immunoprecipitation with antibody specific to HBsAg, and the immune complexes were examined by core protein and HA specific immunoblotting. As shown in Fig. 4, core protein was indeed efficiently coprecipitated with HBsAg. However, the core protein obtained as a 21-kDa band had no change after BM601 treatment. HA-tagged surface protein expressed by pHA-HBs transfected or pHA-HBs plus pHBc cotransfected Huh7 cells could be observed by HA antibody followed by immunoprecipitation of HBsAg antibody while the input S protein (24 kDa) could not be directly detected because it possessed the same molecular weight as the light chain of the antibody. The amount of precipitated HA-tagged S protein in pHA-HBs transfection was larger than the pHA-HBs plus pHBc cotransfection, which coincident with the amount of the HA-tagged S protein expressed. To note, core protein failed to interact with surface protein in pHA-HBs and pHBc cotransfected Huh7 cells, likely due to the lack of L protein as intermediate between the two proteins.

3.5. BM601 interfered with the surface protein convergence in trans Golgi apparatus

The HBV envelope protein does not only exist in plasma but also buds efficiently from intracellular through post-ER pre-Golgi intermediate compartment (Huovila et al., 1992). SVP assembly is initiated by integration of surface protein in ER membrane, where the filaments composed of transmembrane dimmers augment and are transported to the ERGIC. Upon conversion into

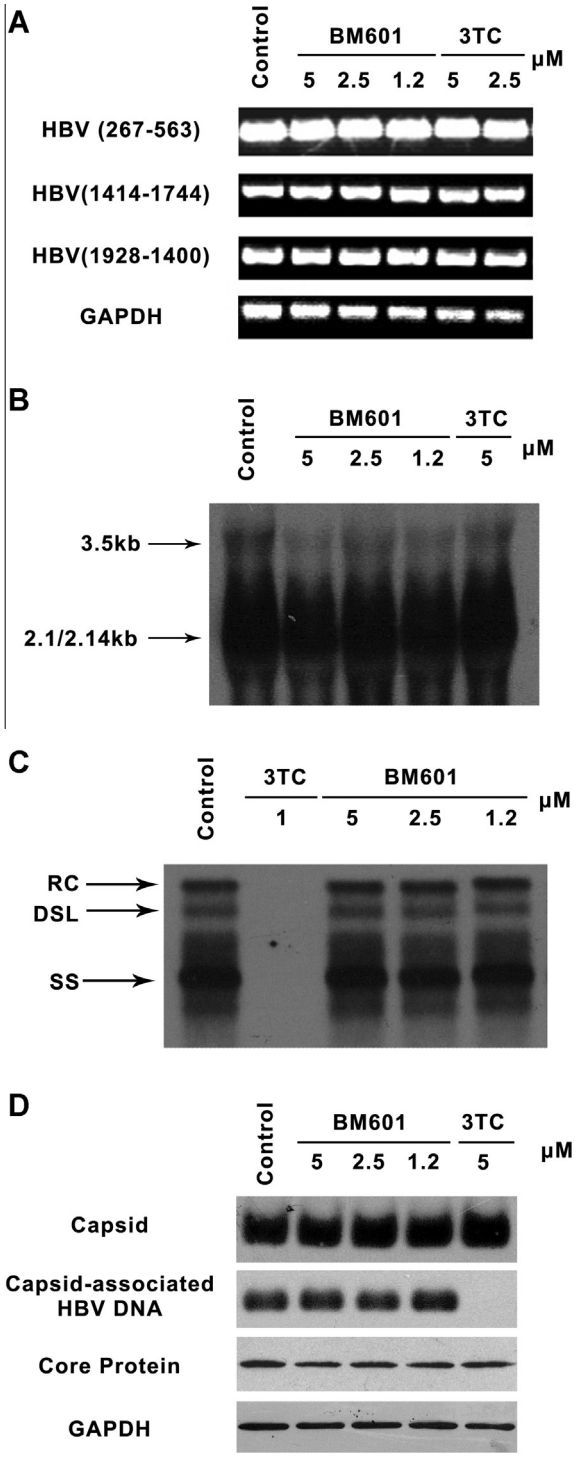

Fig. 2. HBV RNA transcription, HBV DNA replication, core particle encapsidation or core protein production were not affected by BM601. (A and B) Total RNA was isolated from HepG2.2.15 cells treated by BM601 for 8 days. Results from northern blotting and RT-PCR showed no apparent difference in the 3.5 kb/2.4 kb/2.1 kb HBV specific transcripts between control cells and cells treated with BM601. (C) HBV intracellular core-associated DNA from HepG2.2.15 cell lysates was analyzed using Southern blotting with a DIG-probe. Three intracellular HBV DNA forms, relaxed circular (rc) DNA, double-stranded liner (dl) DNA and single-stranded (ss) DNA, did not change after treatment of BM601, but dramatically reduced after 3TC (1 μM) treatment. (D) HepG2.2.15 cell lysates containing core particles were separated by Agarose Gel. BM601 affected neither capsid the formation of nucleocapsid nor the quantity of capsid-associated DNA. Core protein was detected by western blotting. BM601 did not inhibit viral core protein synthesis (upper panel). GAPDH (lower panel) level was used as loading control.

spheres, SVPs are then sent through the constitutive secretory pathway, transported to Golgi complex where Surface proteins are glycosylated. To explore the possibility that the effect of BM601 on virion secretion is related to the translocation of surface protein, we compared surface protein intracellular localization before or after BM601 treatment by performing indirect immunofluorescence analysis. Without BM601 treatment, the surface proteins diffused partly in cytoplasma, and only a small part of punctate structures co-localized with ER (Fig. 5A), while larger part co-localized with *trans*-Golgi ready to secrete out (Fig. 5B). By contrast, after BM601 treatment, the surface proteins distributed more diffusely in cytoplasm, without co-localizing with the polarized Golgi structure staining (Fig. 5B). So we concluded that BM601 prevented the co-localizing of HBV surface proteins in the *trans*-Golgi. It was conceivable that surface proteins were not delivered to Golgi apparatus, blocked by BM601 from moving forward to the following secretion pathways.

3.6. BM601 did not trigger cellular stress response

As previously described, BM601 reduced the extracellular HBsAg and HBV virion significantly, but did not influence the HBV lifecycle before secretion, which implied that BM601 was a secretion inhibitor. However, whether BM601 would produce cellular reactive oxygen species (ROS), trigger ER Stress or affect the secretion of host cellular proteins was still unknown. We detected the ROS production using DCFH-DA detection assay, which could be oxidized by ROS to the highly fluorescent product dichlorofluorescein (DHF). Results showed that BM601 did not induce ROS compared to vehicle, while Rosup raised the ROS dramatically (Fig. 6A). On the other hand, GRP78 accumulation reflecting the incidence of ER stress (Lee, 2005) was not observed after BM601 treatment (Fig. 6B). Consistent with the previous report, N-glycosylation inhibitor tunicamycin and disulfide reducing agent DTT treatment of HepG2.2.15 cells induced expression of endogenous ER stress marker GRP78 (Miyata et al., 1998; Hiramatsu et al., 2006). We subsequently measured whether BM601 had effect on the secretion of host cellular proteins. Secreted alkaline phosphatase (SEAP) is a well established assay for monitoring of protein processing and ER stress in mammalian cells (Berger et al., 1988; Hiramatsu et al., 2006). Results showed that BM601 did not inhibit SEAP secretion compared to secretion inhibitor Monensin (Griffiths et al., 1983) (Fig. 6C). Alphafoetoprotein (AFP) and alpha-1 antitrypsin (AAT) are useful tumor marker in patients with hepatocellular carcinoma (Naitoh et al., 1999) and are sustained secreted by hepatoma cell lines such as HepG2.2.15. We detected the extracellular AFP, AAT as well as both hepatitis B virus antigens. Results shown in Fig. 6D suggested that BM601 did not affect the AFP and AAT secretion while had potent inhibitory effect on HBsAg. It is notable that HBeAg secretion was not reduced after BM601 treatment. These results implied that BM601 interfered with the secretion pathway evolved in hepatitis B virus and SVP without affecting the cellular functions.

4. Discussion

The properties of benzimidazole and its derivatives had been studied for over more than one hundred years (Spasov et al., 1999). They had been reported to be effective in many biological profiles such as antibacterial (Gunes and Cosar, 1992; Kucukbay et al., 2001), antitumor (Antonini et al., 1988; Hranjec et al., 2010; El-Nassan, 2012), antiviral (O'Sullivan and Ludlow, 1972; Tonelli et al., 2008), and antiallergic activities (Awata et al., 1989; Nakano et al., 1999). In our previous studies, we first reported that

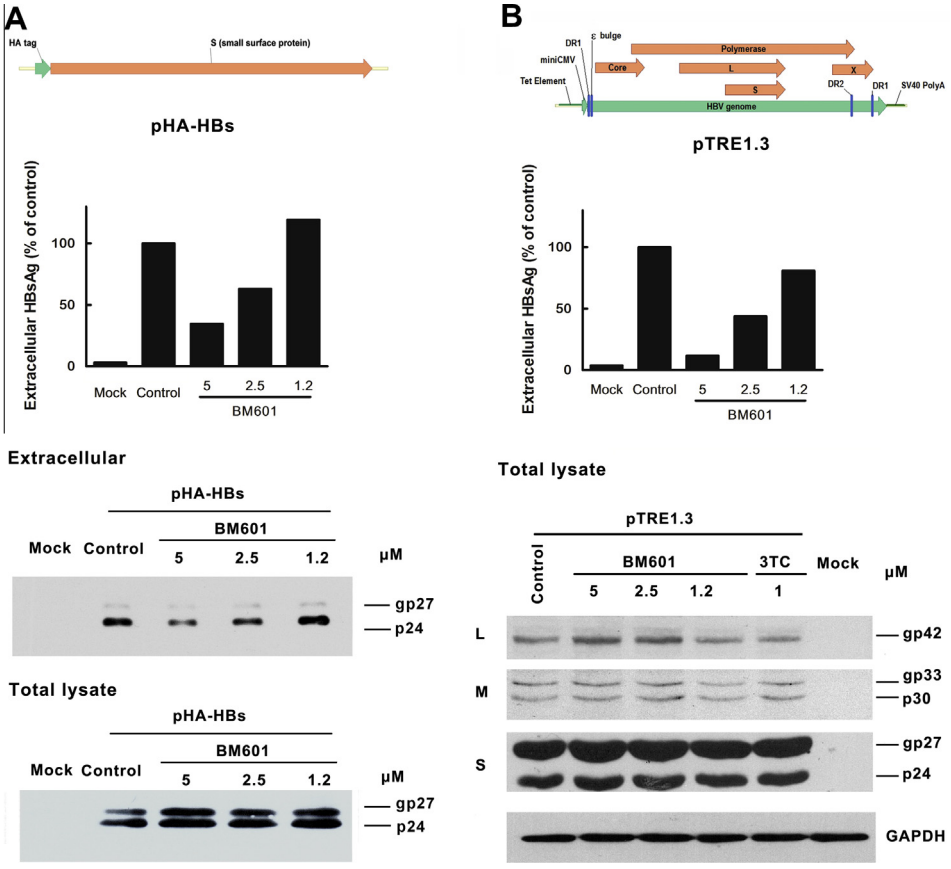

Fig. 3. BM601 inhibited S surface protein secretion, but did not change the ratio of three kinds of surface proteins. (A) pHA-HBs was transfected to Huh 7 cells, after treated with BM601, surface protein was detected by ELISA and Western blot. The total secreted HBsAg was reduced after BM601 treatment. BM601 had no effect on intracellular S surface protein production, but decreased both unglycosylated (24-kDa) and glycosylated (27-kDa) S surface protein secretion. (B) Diagram of pTRE1.3 construct used in this study. Only in the presence of tetracycline the full length DNA under control of P_{tight} promoter could be transcribed. The L, M, S surface protein had its own promoter and would be continuously expressed. Huh7 cells were transfected with pTRE1.3; the extracellular HBsAg was detected by ELISA while the total lysate was determined by Western blot. The same blot was probed for L/M/S surface protein and GAPDH (to serve as loading control). Though BM601 dramatically reduced the secreted HBsAg in the supernatant, the production of L, M and S surface protein did not change after BM601 treatment.

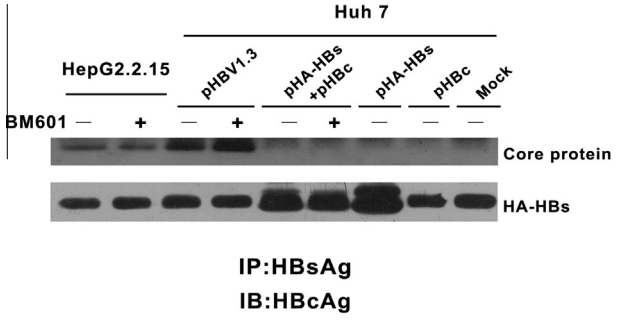

Fig. 4. BM601 did not affect the interaction between surface protein and core protein. Cell lysates were precipitated by horse anti-HBsAg polyclonal antibody, and then core protein was detected by Western blotting. Cells were previously treated with BM601 at concentration of 5 μM or vehicle. The core protein precipitated did not change after BM601 treatment. pHA-HBs, a plasmid harboring the S surface protein except for an C-terminal insertion of a HA tag. pHBc, a plasmid expressing the full-length Cp185 protein.

benzimidazole derivatives possessed anti-hepatitis B virus activity (Li et al., 2006). However, how benzimidazole derivatives affect HBV lifecycle was still illusive. Here we reported the anti-HBV mechanism of a benzimidazole derivative, BM601. BM601 showed a unique pattern of action with potent inhibition activity toward HBV secretion as well as SVPs secretion. In cell culture system, it possessed an EC_{50} on HBV DNA and HBsAg in low micromolar ranges, with selective index large enough to suggest that the inhibitory activity of BM601 was not related to nonspecific cellular metabolism defect caused by cytotoxic effect. BM601 had no effect on HBV DNA replication, RNA transcription or virus protein production. The secreted SVPs reduced significantly upon sole surface protein expression. Surprisingly, BM601 did not affect surface protein production as well as its interaction with nucleocapsids. It implied that BM601 might work on secretion pathway. Results from immunofluorescence revealed that BM601 changed the location of surface proteins in *trans*-Golgi network, that might be the mechanism of action how BM601 inhibited virion and HBsAg secretion.

The inhibitory effect of BM601 on HBsAg secretion raised a question that whether the antiviral activity of BM601 was mediated through the cellular function damage. We did not observe any cytotoxicity after BM601 treated for 8 days at concentration of 10 μM. What's more, the ROS production was not raised, the GRP78 were not accumulated in ER and the SEAP secretion was not affected, so was the secretion of AFP and AAT. All the results showed that the cellular secretion function was not inhibited. The EC_{50} of BM601 on HBsAg in HepG2.2.15 was 1.5 μM, though the inhibitory of BM601 on CMV promoted HA-HBs is weakened. Presumably, that was caused by much larger quantity of HA-HBs expression and shorter time of BM601 treatment since BM601 worked in the late process of HBsAg secretion.

There were some reports implying that SVP secretion was independent to virion secretion (Chua et al., 2006; Lambert et al., 2007;

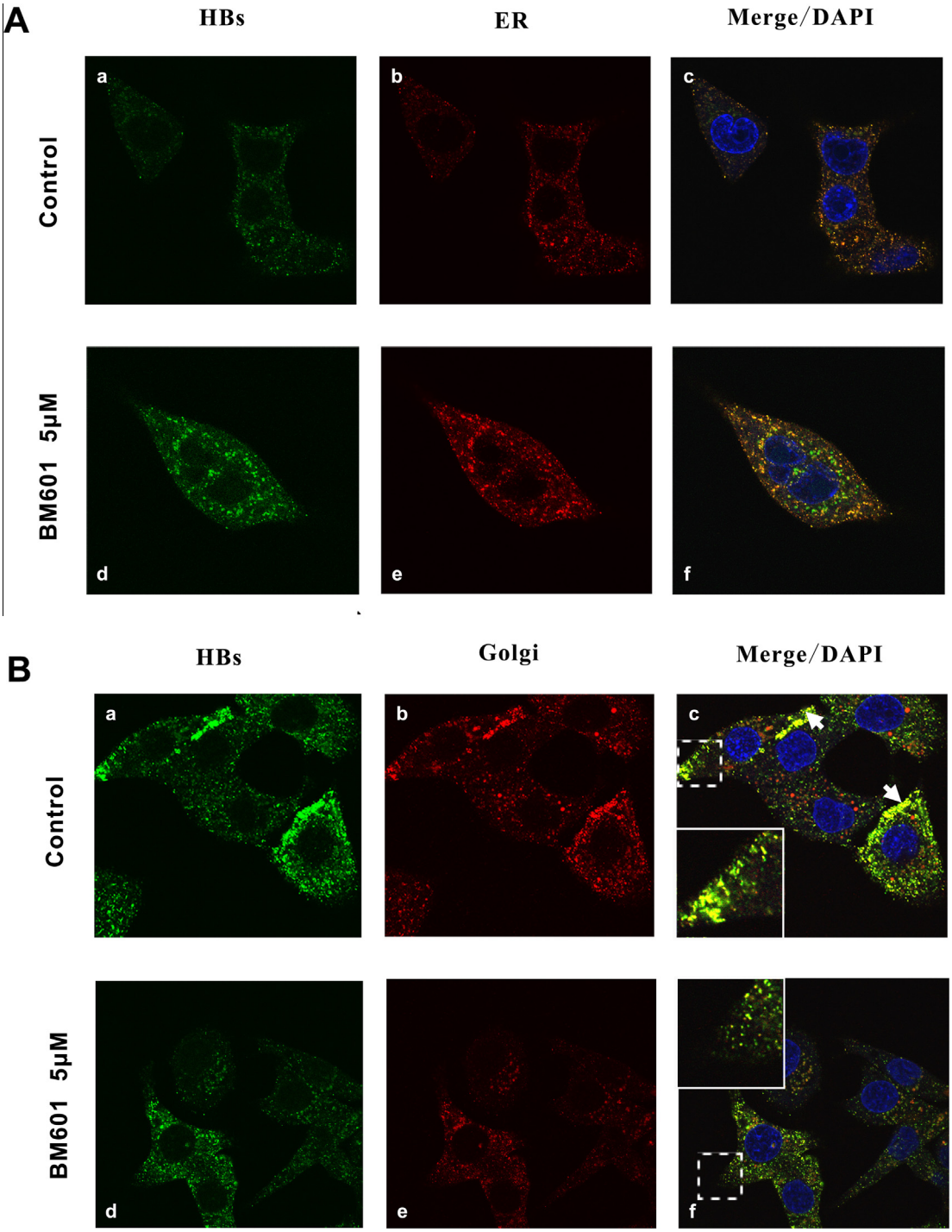

Fig. 5. BM601 abolished the surface protein aggregation in polarized Golgi apparatus. HepG2.2.15 was planted on coverslips for 12 h followed by 72 h of BM601 treatment to 90% confluence. Cells were harvested and fixed with 4% paraformaldehyde, and stained for HBsAg (green) and Calnexin (ER specific membrane marker, red) (A) or Giantin (Golgi specific membrane marker, red) (B). Consistent with previous studies, HBsAg displayed partly diffused and granular staining pattern in cytoplasm. After BM601 treatment, the surface proteins were more diffusely distributed in cytoplasm, without co-localizing with the polarized Golgi structure staining. The blue color of the nucleus was stained with DAPI.

Patient et al., 2007; Watanabe et al., 2007; Garcia et al., 2009), however, mutants like I110M, G119E, and R169P impaired virion secretion through reduction of the small envelope protein (Ito et al., 2010). Two conserved residues mutation reported in HBsAg, M75 and M103, attenuated HBsAg secretion and suppressed HBV genome replication without compromising the overlapping p-gene product, indicating that surface proteins were involved in regulation of HBV genome replication (Qiu et al., 2011). In our study, we assumed that the secretion of both mature virions and SVPs were blocked by BM601 through a shared pathway or that they affected each other in a manner which needed further exploration. On the other hand, we demonstrated that BM601 did not affect the interaction between surface protein and core protein through the co-immuoprecipitation assay. Actually, sole co-immunoprecipitation assay cannot fully reveal the budding of intracellular HBV virions into intracellular lumen or vesicles. So more experiments will be

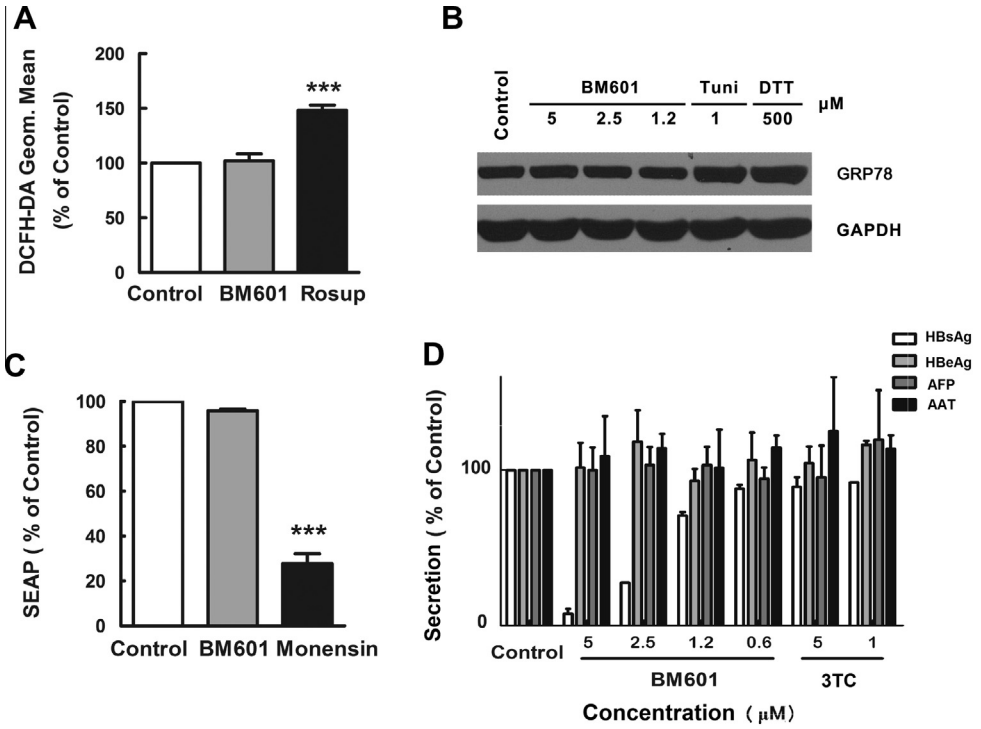

Fig. 6. BM601 did not induce ROS generation, trigger ER stress or influence host cellular protein secretion. (A) HepG2.2.15 cells were treated with BM601 (5 μM) or Rosup (20 μg/ml). A compound mixture was used as positive control. Intracellular superoxide was detected by DCFH-DA or DCF-based measurements. (B) HepG2.2.15 cells were treated with indicated concentration of BM601, Tunicamycin or DTT for 24 h. The total lysates were used for western blotting for GRP78 and GAPDH detection. GRP78 levels increased significantly in tunicamycin- and DTT-treated HepG2.2.15 cells while remain the same as control in BM601 treated HepG2.2.15 cells. (C) Huh 7 cells were treated with BM601 or Monensin for 3 days after transfection with pSEAP2-Basic Vector plasmid. The supernatant SEAP secretion was not changed after BM601 treatment while dropped dramatically when treated with secretion inhibitor Monensin (2 μM). (D) HepG2.2.15 cells were treated with indicated concentration of BM601 for 7 days, the supernatant HBsAg, HBeAg as well as AFP and AAT were detected through ELISA assay. The HBsAg secretion was inhibited by BM601 in a dose-dependent manner while the secretion of HBeAg, AFP and AAT was not affected after BM601 treatment.

needed in the future to address BM601's effects on budding process. ROS aggravated the hepatic inflammation that might contribute to the early stage of HBV-associated carcinogenesis (Lim et al., 2010). To exclude this possibility, we detect ROS production and ER stress after BM601 treatment. BM601 induce neither ROS nor ER stress, suggesting that no extra surface protein accumulated in ER. This was also consistent with our confocal results. The translocation of surface protein in ER enabled the surface protein process in SVP assembly as well as virion envelopment. The SVPs organized in ERGIC and transport through constitutive secretory pathway traversing the trans-Golgi. Immunofluorescence analysis suggested BM601 accounted for the phenomenon that the aggregation of surface protein in *trans* Golgi complex was aborted. Blocked in the secretory pathway, SVPs cannot exit from hepatocytes with the consequence that the supernatant HBsAg reduced after BM601 treatment. Taken together, our current hypothesis of the mechanism of BM601 anti-HBV action is that BM601 might interfere with the secretion of mature virions and SVPs through interfering surface protein aggregation in *trans* Golgi apparatus.

The secretory pathway of HBV has long been a potential target for novel anti-HBV drugs, which may offer the prospect of markedly improving the response to current therapies. The iminosugar derivatives of butyldeoxynojirimycin and related glycolipids, the inhibitor of α-glucosidase which altered specific steps in the N-linked glycosylation pathway, exerted inhibitory effect on the secretion of HBV surface protein (Block et al., 1994, 1998) and other virus glycoproteins (Block and Jordan, 2001). Root extract of Boehmerianivea (BNE) could significantly accumulate viral core and large surface proteins accompanied with their encapsidated viral DNA within cell through the reduction of ER chaperone GRP78 (Huang et al., 2009). Llama-derived single-domain intrabodies were the first intrabodies that inhibited viral secretion when expressed and retained in ER (Serruys et al., 2009). Varying from the mechanism of them, BM601 specifically inhibits HBsAg secretion without affecting the glycosylation of all three kinds of surface proteins or inducing any ER chaperone variation. Triazolo-pyrimidine derivatives (Yu et al., 2011) were also reported as specific inhibitors of HBsAg secretion independent of viral genomic replication, but they exhibited slightly accumulation of intracellular L and M, showing a different mechanism from BM601 (Dougherty et al., 2007). Therefore BM601 takes a totally new mode of action as a secretion inhibitor.

Another implication for the utility of BM601 may be as a tool drug uncovering novel aspects of HBV secretion pathways. Pathways in budding and release of SVPs, virions, and naked capsids remain elusive. Integrating S in the ER membrane initiates SVP assembly. Then transmembrane dimmers are delivered via vesicles to the ER-Golgi intermediate compartment (ERGIC) where complex S structures are remodeled to form spheres and filaments (Huovila et al., 1992; Patient et al., 2007). The SVPs were exported through the constitutive secretory pathway. Unlike the SVPs, the virions used multivesicular body to transport to the budding site, recruiting the endosomal sorting complex required for transport (ESCRT) system (Lambert et al., 2007; Rost et al., 2006; Li et al., 2011; Prange, 2012). However, how Golgi apparatus function involvs in virion maturation is not known yet, probably because for decades, it has been assumed that the secretion of HBV virions can be reflected by the budding of SVPs. Overall, it cannot be denied that both secretion pathways share common elements. Interfering processes involving in constitutive secretory pathway or ESCRT

system, BM601 may provide more information on HBV and SVPs secretion.

As a novel chemical entity that is able to inhibit HBV and HBsAg secretion, BM601 is now under structural optimization and SAR study. A series of BM601 analogs with different structural modifications were tested in HepG2.2.15. Though the potency of the series was not improved compared to BM601, some of them maintained the activity. It suggests that benzimidazole derivatives indeed possess the HBV-secretion inhibitory activity. In conclusion, this study shows that benzimidazole derivatives can inhibit secretion of both HBV and HBsAg, throwing light on the diverse usage of benzimidazole derivatives and providing more possibility for HBV management.

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