Hepatitis B e Antigen Synthesis is Inhibited by a Galactosylated Arginine-Rich Macromolecule Via Asialoglycoprotein Receptor

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Abbreviation:
HBV: hepatitis B virus; HBeAg: hepatitis B e antigen; HBsAg: hepatitis B surface antigen; CMK: decanoyl-RVKR-chloromethylketone; D6R: hexa-D-arginine; D9R: nona-D-arginine; ASGP-R: asialoglycoprotein receptor; gal: galactosylated; FPd: furin prodomain; BSA: bovine serum albumin; NTCP: Sodium taurocholate cotransporting polypeptide; PCR: polymerase chain reaction; GEeq: genome equivalent; HAS: human serum albumin; PTB: prothrombin; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; ALT: alanine aminotransferase

1. Abstract

1.1. Aims: The poor prognoses of hepatitis B virus (HBV) infection are correlated with hepatitis B e antigen (HBeAg). Current antiviral therapy does not directly interfere with HBeAg synthesis. HBeAg formation depends on proprotein convertase furin. To block HBeAg synthesis on purpose, galactosylated arginine-rich macromolecular furin inhibitors were developed and evaluated.

1.2. Methods: Galactosylated macromolecular inhibitors, arginine-rich core peptides with N-terminal galactosylation and C-terminal connection with bovine serum albumin, were designed and synthesized to be hepatocyte-targeting and the trans-Golgi network furin-accessible via the asialoglycoprotein receptor (ASGP-R). Their efficiency and other characteristics were evaluated in HBV-transformed and -infected model cells.

1.3. Results: HBeAg-inhibitory efficiency of the core peptides was not affected by galactosylation. In the successfully-prepared galactosylated arginine-rich macromolecular furin inhibitor, each molecule of bovine serum albumin combined with about twenty core peptides. The furin inhibitor took effects slowly, but the eventual efficiency increased to five times and had better carry-over effect. The new inhibitor entered cells and took effects in an ASGP-R-dependent manner and the high effect of the new inhibitor did not lead to increasing interferences with the albumin and prothrombin secretions in HBV-transformed HepG2.2.15 cells. Furthermore, the new inhibitor also significantly inhibited HBeAg secretion and promoted the cell surface expression of HBeAg precursor without substantial side-effects in HBV-infected model cells.

1.4. Conclusion: Furin-inhibitory peptides delivered via ASGP-R were effective and nontoxic. The macromolecular inhibitor, due
to the hepatocyte-targeting and furin-accessible abilities, may be a promising candidate to be used to acquire HBeAg seroconversion efficiently in the future.

2. Introduction

Hepatitis B virus (HBV)-infected patients with persistent existence of hepatitis B e antigen (HBeAg) usually have a poor prognosis [1]. Frankly, those patients with HBeAg-defective HBV infection (HBeAg-negative chronic hepatitis B) also have a poor prognosis [2]. However, the defective viruses derive from wild-type (HBeAg synthesis-competent) strains, usually during the late stage of infection, implying that the successful control of infection in HBeAg-positive stage may reduce the overall risks. Although hepatitis B surface antigen (HBsAg) clearance or seroconversion is thought as the ideal end-point of antiviral therapy, to realize HBeAg seroconversion is their prerequisite. Unfortunately, current antiviral options of recombinant interferon α and nucleotide/nucleoside analogues have a low rate of HBeAg seroconversion due to lack of direct inhibitory effect on HBeAg synthesis [3, 4], urging for novel combinations or restoration of immune responses to cure HBV infection [5-7]. In pathogenesis, HBeAg induces host immunotolerance [8, 9], suggesting that the blockage of HBeAg synthesis on purpose may be favorable for HBeAg seroconversion and host antiviral immune restoration.

HBeAg synthesis begins with the precore protein that is encoded by the C open reading frame of viral genome. The nascent peptide during the translation of the precore protein is directed to endoplasmic reticulum by a 19-amino acid signal peptide in its N-terminus. After the signal peptide is removed, HBeAg precursor is generated and transported to the trans-Golgi network for further proteolysis by proprotein convertase furin in arginine-rich domains of pre-core minus to generate mature HBeAg [10, 11]. Furin cycles among the trans-Golgi network, cell surface and endosomes. Our previous studies have shown that a functional single nucleotide polymorphism in the P1 promoter of furin gene correlates with the outcome of HBV infection [12], and furin inhibitors not only reduce the secretion of HBeAg, but also increase the expression of HBeAg precursors on cell surface in model cells [13, 14]. Interestingly, the lack of surface expression of HBeAg precursor is the important mechanism of persistent existence of HBeAg-defective HBV infection [15]. Therefore, furin inhibition may be a new approach to acquisition of HBeAg seroconversion and prevention of HBeAg-negative chronic hepatitis B.

Furin recognizes the arginine-rich domains of proteins. Furin inhibition in laboratory is usually conducted using small artificial arginine-rich peptides decanoyl-RVKR-chloromethylketone (CMK), hexa-D-arginine (D6R) and nona-D-arginine (D9R). CMK has been shown to reduce HBeAg secretion in cell-based tests [10, 11]. However, our previous studies have shown that CMK leads to HBV replication enhancement (an effect opposite to treatment) by off-targeted inhibition of the trypsin-like activity of cellular proteasomes [13, 16]. Compared with CMK, D6R and D9R are more effective in vitro [17]. They are also less toxic and are commonly used in the in vivo studies [18]. Our previous study shows that D6R inhibits HBeAg secretion without HBV replication enhancement, but harms the cell membrane and is not as effective as CMK, perhaps due to the poor permeability [13]. In addition, the conditional knockdown of furin in lymphocytes leads to autoimmune diseases in mice [19]. Therefore, no furin inhibitors up to now meet clinical needs with respect to efficacy and side effects and new inhibitors had better possess hepatocyte-targeting and the trans-Golgi network furin-accessible abilities. The selective delivery of drugs to hepatocytes can be realized via the asialoglycoprotein receptor (ASGP-R) [20, 21]. Excitingly, this manner delivers the inhibitors into the endosomal-lysosomal compartments from where the basic inhibitory elements may be translocated to the trans-Golgi network.

In this study, we designed and prepared a galactosylated macro-molecular furin inhibitor, galactosylated arginine-rich furin prodomain-linked bovine serum albumin (gal-FPd-BSA). This new inhibitor was hepatocyte-targeting and furin-accessible and showed an enhanced efficiency and reduced side-effects, highlighting a clinical potential in promotion of HBeAg seroconversion in the future.

3. Material and Methods

3.1. Furin Galactosylated Macromolecular Inhibitor Design and Preparation

The galactosylated macromolecular inhibitors (gal-FPd-BSA and gal-D9R-BSA) were developed using furin prodomain (N'-AKRRT-KR-C') and D9R as basic cores, respectively. The core peptides were galactosylated in the N-terminal and subsequently linked with BSA in C-terminus (Figure 1). Peptide syntheses were initiated by a galactosylated threonine and ended by a cysteine. The galactosylated peptides were linked with lysine residues of BSA by the crosslinker succinimidy 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (ThermoFisher Scientific, Shanghai, China). Peptide syntheses and BSA linkages were conducted in Chinapeptides co., Ltd (Shanghai, China).

Figure 1: The diagrammatic structure of galactosylated macromolecular furin inhibitors. BSA, bovine serum albumin; gal, galactosylated; D9R, nona-D-arginine; FPd, furin prodomain.
3.2. Other Peptides and BSA Conjunctives
To evaluate the influences of galactosylation and BSA conjunction on the inhibitory effects of the core peptides and the dependence of ASGP-R, other peptides with (gal-FPd and gal-D9R) or without galactosylation (FPd and D9R), BSA conjunction without galactosylation (FPd-BSA) and fluorescein Cy3-labeled conjunctives (Cy3/gal-FPd (1:1) -BSA and Cy3/FPd (1:1) -BSA) were synthesized (Chinapeptides co., Ltd, Shanghai, China).

3.3. HepG2-NTCP Cell Line Construction and HBV Infection
The Gene of human sodium taurocholate cotransporting polypeptide (NTCP) was cloned into pcDNA3.1 as reported [22], and then sub-cloned into lentiviral expression vector pCDH-CMV-EF1-copGFP-T2A-Puro to generate NTCP-lentiviruses. HepG2-NTCP cells were regularly established by infecting HepG2 cells with the viruses. The infection was monitored by examining the copGFP using fluorescent microscopy. The transcription was evaluated using reverse transcription-polymerase chain reaction. HBV inoculum was prepared from a 24-years-old female patient with serum HBV DNA of 3.5×10^7 copies/mL (genotype C) by a method of PEG8000/NaCl precipitation. HepG2-NTCP cells were infected with HBV inoculum at 100 genome equivalent (GEq)/cell in the presence of 4% PEG8000 for 16 hours.

3.4. Cell Culture and Furin Inhibitor Treatments
HepG2.2.15, HepG2 and HepG2-NTCP cells were grown in Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum. For HepG2.2.15 and HepG2-NTCP cells, the medium was supplemented with 380 µg/mL of genetin and 2 µg/mL of puromycin, respectively. The sustaining culture medium (2% fetal bovine serum) was used when cells were at confluency growth. To evaluate the effects of furin inhibitors on HBeAg secretion, 100 µmol/L D6R (EMD Millipore Corporation, Billerica, MA, USA) was served as positive control. Furin inhibitory peptides, galactosylation (FPd and D9R), BSA conjunctive without galactosylation (FPd-BSA) and fluorescein Cy3-labeled conjunctives (Cy3/gal-FPd (1:1) -BSA and Cy3/FPd (1:1) -BSA) were synthesized (Chinapeptides co., Ltd, Shanghai, China).

3.5. Western Blot Analysis
BSA conjunctives, human serum albumin (HSA) and prothrombin (PTB) were detected using Western blot analysis. The harvested media were concentrated to one tenth in volume using centricon-10 microconcentrator (Millipore Corporation, Billerica, MA, USA). BSA conjunctives and the concentrated media were regularly separated and transferred into polyvinylidene fluoride membranes (Millipore Corporation). Immunoblot analysis was performed using polyclonal antibodies to HSA (Abcam, Cambridge, UK) or PTB (Abcam) and enhanced chemiluminescence reagent (Invitrogen Corporation, Shanghai, China).

3.6. Flow Cytometry
To evaluate the ASGP-R dependence of gal-FPd-BSA, cells were stained with Cy3-labelled gal-FPd-BSA or FPd-BSA for 30 minutes. To evaluate the influence of gal-FPd-BSA on the cell surface expression of HBeAg precursor, cells were stained without fixation in tissue culture dishes with the rabbit polyclonal anti-HBc antibody (Dako, Carpinetia, CA, USA) and the Cy3-labelled goat anti-rabbit IgG antibody (Abcam). The above stained cells were gently washed off the plates and analyzed using a FACS LSRII cytometer (Becton Dickinson, San Jose, CA, USA).

3.7. Miscellaneous
HBsAg and HBeAg in media were quantified using commercial kits of chemiluminescence immunoassay (USCNK Life Science Incorporation, Wuhan, China). Cell viability was evaluated using 3-(4,5-di-methyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. The core particles in supernatant were isolated by a method of PEG8000/NaCl precipitation. The supernatant core-associated HBV DNA was quantified using real-time fluorescent PCR (Taan Gene Company).

3.8. Statistical Analyses
The differences in supernatant virions, HBsAg and HBeAg, and the cell viability were analyzed using the Student’s t-test. A P<0.05 was considered statistically significant. All statistical analyses were conducted using SPSS software (version 11; SPSS Incorporation, Chicago, IL, USA).

4. Results
4.1. Galactosylation Does Not Affect the Effects of Arginine-Rich Furin Inhibitors
D6R is common reference furin inhibitor among different laboratories. D6R of 100 µmol/L has been tested to inhibit HBeAg secretion significantly in HepG2.2.15 cells [13]. Thus, D6R was here used as a reference to evaluate the influence of galactosylation on the efficacy of these core peptides, FPd and D9R. These peptides and their galactosylated forms (gal-FPd and gal-D9R) were tested in HepG2.2.15 cells with final concentration of 100 µmol/L. As shown in Figure 2D, D6R and those artificial peptides with or without galactosylation all inhibited the levels of supernatant HBeAg (Figure 2A) and HBsAg (Figure 2B) without affecting the cell viability (Figure 2C). No significant difference was found between the interesting peptides and their galactosylated forms.

4.2. BSA Conjunction Enhances the Inhibitory Efficiency on HBeAg Synthesis
When BSA conjunctives were evaluated by gel electrophoresis (Figure 3A), the gal-D9R was found to be unable to bind BSA efficiently. Since three re-syntheses showed similar results, the development of D9R-BSA conjunctive was canceled. Fortunately, the gal-FPd-BSA was successfully constructed. Its molecular weight was about 95...
~100 kDa (Figure 3A). Based on the change of molecular weight, each BSA (66.4 kDa) molecule averagely bound 20 molecules of gal-FPd (1.54 kDa), namely, 5 µmol/L gal-FPd-BSA was equivalent to 100 µmol/L gal-FPd. When this equivalent relationship was used in time-effect analyses, though slightly weaker in the beginning (24 hours), gal-FPd-BSA inhibited HBeAg secretion significantly stronger than gal-FPd at last (72 hours) (Figure 3B). The cell viability was not affected by both gal-FPd-BSA and gal-FPd (Figure 3C). When cells were treated for 72 hours, the dose-effect studies showed that BSA conjunction reduced the cell toxicity at high concentration (Figure 3D), and the inhibitory efficiency significantly enhanced at the same equivalent doses (calibrated against cell viability in high equivalent doses) (Figure 3E). It was obvious that BSA conjunction reduced equivalent dose to one fifth, namely increased efficiency to five times. Furthermore, BSA conjunctive had a much better carry-over effect (Figure 3F).

Figure 2: The influence of galactosylation on the effects of furin inhibitory peptides. HepG2.2.15 cells were cultivated with or without 100 µmol/L peptide inhibitors (D6R, hexa-D-arginine; D9R, nona-D-arginine; FPd, furin prodomain; gal, galactosylated;) for 48 hours. HBsAg and HBeAg in media were detected using ELISA. Cell viability was evaluated by MTT analysis. * P<0.05, ** P<0.01, NS, no statistical significance. The supernatant (A) HBeAg and (B) HBsAg were inhibited by the peptide inhibitors without affecting (C) the cell viability.

Figure 3: The influence of BSA conjunction on the effect of furin peptide inhibitor. HepG2.2.15 cells were cultivated with or without 100 µmol/L gal-FPd (gal, galactosylated; FPd, furin prodomain) or 5 µmol/L gal-FPd-BSA (BSA, bovine serum albumin) for 72 hours unless mentioned specifically. HBeAg in media was detected using ELISA. Cell viability was evaluated by MTT analysis. * P<0.05, ** P<0.01. (A) The molecular weight of gal-FPd-BSA was 95 ~100 kDa, indicating that each BSA (66.4 kDa) molecule averagely bound 20 molecules of gal-FPd (1.54 kDa). Compared with equivalent gal-FPd, gal-FPd-BSA (B) was less efficient in 24 hours, but more efficient in 72 hours in reduction of supernatant HBeAg (C) without cell toxicity, and was of (D) less cytotoxicity at higher concentration, (E) higher efficiency (imaginary box, calibrated against cell viability; NS, no statistical significance) in HBeAg inhibition and (F) better carry-over effect.
4.3. Galactosylated Macromolecule Takes Effects in a Manner of ASGP-R Dependence

BSA conjunction changed the performances of gal-FPd by delaying taking effect, but increasing the eventual efficacy and the carry-over effect, which are in concordance with the internalization of the ligand-ASGP-R complex with a prolonged half-life of 5 days [23], suggesting that the galactosylated macromolecule entered cells via ASGP-R. However, those might also be resulted from the direct effect on cell surface furin due to the enlargement of the molecular weight. To rule out the possibility, we synthesized the BSA conjunction without galactosylation (FPd-BSA). Both conjunctives were successfully synthesized (Figure 4A). After balancing their concentrations carefully, we found that the galactosylation significantly enhanced the inhibitory efficiency for more than five times in HepG2.2.15 cells (Figure 4B). In flow cytometry, the Cy3-labelled gal-FPd-BSA and FPd-BSA entered the cells in dose-dependent and -independent manners, respectively (Figure 4C, upper left). The gal-FPd-BSA entered the cells more efficiently, especially at the medium concentration (500 µmol/L) (Figure 4C, upper right). The equivalent dosage was reduced to about one fifth (Figure 4C, bottom), indicating that the permeability of the galactosylated one increased to five times. Moreover, the enhancement was abrogated by 2 mmol/L D-galactose (Figure 4C, bottom right). The results ascertain that gal-FPd-BSA takes effects in an ASGP-R-dependent manner.

4.4. Galactosylated Macromolecule Did Not Affect Cell Function in HepG2.2.15 cells

D6R at common doses does not affect the cell functions, but increases the alanine aminotransferase (ALT) in culture media [13]. Compared with peptide inhibitors, the BSA conjunction showed much better drug performances including the reduction of cell toxicity. However, the delivery via ASGP-R may cause the accumulation of inhibitor in the trans-Golgi network to impair the cell normal secretory functions. For this reason, the effects of BSA conjunction on cell functions were also tested here. The gal-FPd-BSA at effective concentrations did not affect the ALT level in culture media (Figure 5A) and the syntheses of HSA and PTB in HepG2.2.15 cells (Figure 5B).

**Figure 4:** Galactosylated macromolecule takes effects in a ASGP-R-dependent manner. (A) The molecular weights of FPd-BSA (FPd, furin prodomain; BSA, bovine serum albumin) and gal-FPd-BSA (gal, galactosylated) were comparable. (B) HepG2.2.15 cells were cultivated with or without various concentrations of FPd-BSA or gal-FPd-BSA for 72 hours. HBeAg in media was detected using ELISA. **P<0.01. The galactosylated conjunction was much more efficient (5~25 times) in inhibition of supernatant HBeAg. (C) Cy3/FPd-BSA and Cy3/gal-FPd-BSA entered the cells in different manners. Cy3/gal-FPd-BSA entered the cells with a higher efficiency (5 times), which was blocked by D-galactose.
Figure 5: Potential side-effects of galactosylated macromolecule in HepG2.2.15 cells. The cells were cultivated with or without 100 μmol/L D6R (hexa-D-arginine), 100 μmol/L gal-FPd (gal, galactosylated; FPd, furin prodomain) or 5 μmol/L gal-FPd-BSA (BSA, bovine serum albumin) for 72 hours. ALT, alanine aminotransferase. Human serum albumin (HSA, 66.5 kDa) and prothrombin (PTB, glycosylated, 68.9 kDa; non-glycosylated, 66.0 kDa) were detected using Western blot analysis. * \( P<0.05 \), ** \( P<0.01 \). The gal-FPd-BSA did not affect (A) the ALT level in culture media and (B) the secretions of HSA and PTB.

4.5. Galactosylated Macromolecule is Effective in HBV-Infected Model Cells

HepG2.2.15 is a transfected HBV cell model. To evaluate whether BSA conjunctive inhibit HBeAg synthesis in HBV-infected cell model, HepG2-NTCP cells infected with HBV was employed. When HepG2-NTCP cells were infected with HBV for 12 days and treated with or without conjunctive for additional 3 days, 5 μmol/L gal-FPd-BSA did not affect the cell viability (Figure 6A) and ALT (Figure 6B), but significantly inhibited HBeAg synthesis (Figure 6C) and up-regulated the expression of precore protein on cell surface (Figure 6D). D6R and CMK have been found to inhibit HBsAg secretion and CMK also undesirably enhance HBV replication in HepG2.2.15 cells [13,14, 24]. Here, gal-FPd-BSA was found to slightly reduce HBsAg in media (Figure 6E), but did not enhance HBV replication (Figure 6F).

Figure 6: The overall effects of galactosylated macromolecule in HBV-infected HepG2-NTCP cells. The cells were infected with HBV (2×10^2 GEq/cells) for 12 days before treating with or without 5 μmol/L gal-FPd-BSA (gal, galactosylated; FPd, furin prodomain; BSA, bovine serum albumin) for 72 hours. Cell growth was analyzed using MTT assay. HBsAg and HBeAg in media were detected using ELISA. HBeAg precursor on cell surface was detected using flow cytometry. HBV virions in media was quantified using qPCR assay. ALT, alanine aminotransferase. * \( P<0.05 \), ** \( P<0.01 \). The gal-FPd-BSA did not affect (A) the cell viability and (B) the ALT level in media, but significantly (C) inhibited supernatant HBeAg and (D) raised the cell surface expression of HBeAg precursor, (E) slightly inhibited supernatant HBsAg and (F) did not affect the virion level in culture media.
5. Discussion

Although targeting furin was thought as a therapeutic strategy for many diseases and the development of new furin inhibitors have become research hotspots for years [17, 18, 25-27], there are no available furin inhibitors in clinical practice. The first obstacle is the inaccessibility of furin that exists mainly in the trans-Golgi network. The second obstacle is the side-effect, such as the cell membrane toxicity of D6R or the off-target effect of CMK to inhibit the trypsin-like activity of cellular proteases and the lethal factor of bacterium Bacillus [13, 28]. The third obstacle is the ubiquitous expression of furin, which suggests that traditional delivery manners may lead to unexpected effects, for example, furin inhibition in lymphocytes leads to autoimmune diseases in mice [10]. In this study, the galactosylated macromolecular furin inhibitor gal-FPd-BSA was found to be effective and nontoxic, supporting that the targeting delivery of the inhibitors to the lysosomal/endosomal compartments of hepatocytes via ASGP-R could break through all above obstacles and highlighting a new way to achieve HBeAg seroconversion in the future.

Furin as a proprotein convertase recognizes and cleaves the consensus sequence RX(K/R)R. Current furin inhibitors CMK, D6R and D9R all derive from this basic prodomain. In this study, we used the candidates FPd (AKRRTKR) and D9R due to their lowest \( K_i \) values (40-100 nmol/L) \textit{in vitro} [29]. D9R was selected also due to its relative resistance to proteases [17, 18]. However, the efficiency of these two peptides in cell-based test was suboptimal (\( K_i \) values, >100 μmol/L, based on HBeAg secretion), likely due to their poor permeability like D6R [13]. Thus, the inhibitory efficiency of the peptide inhibitors is acceptable. The key question is the furin accessibility, namely the efficient delivery of them to the trans-Golgi network where the majority of furin is located.

The ASGP-R is often employed to deliver drugs to hepatocytes in the studies on liver imaging or treatments of hepatitis C and hepatocellular carcinoma [30, 31]. Theoretically, this delivering manner not only declines the toxicity and avoids systematic side-effects, but also increases furin accessibility by overcoming the barriers of cell membrane and the membrane of the trans-Golgi network since the inhibitors is directly delivered to the endosomal-lysosomal compartments of hepatocytes, from where the basic inhibitory elements may be translocated to the trans-Golgi network. However, it requires additional steps to construct macromolecules. Fortunately, the galactosylation of these candidate peptides for furin did not affect the inhibitory effects and gal-FPd-BSA was successfully prepared. The efficiency of this macromolecular inhibitor was five times higher than that of its core unit (gal-FPd), but took effects a little slowly and showed a decrease in cell membrane damage and an enhancement in carry-over effect, suggesting that gal-FPd-BSA did not take effect by inhibition of furin on cell surface as peptide inhibitors and in concordance with that the ligand-ASGP-R complex is usually internalized with a prolonged half-life of 5 days [23]. In flow cytometry, it was reasonable that the galactosylated macromolecular inhibitor entered cells in a dose-dependent manner. The non-galactosylated one, however, seemed to enter cells also efficiently at low or high concentrations. The possible explains are the direct combination with furin on cell surface at low concentration and the passive diffusion due to cell membrane damage at high concentration. Therefore, gal-FPd-BSA was successfully delivered to the cytoplasm, perhaps to the intracellular compartments of hepatocytes via the ASGP-R.

HBV-infected HepG2-NTCP cell model is highly praised currently since it more accurately mimics the HBV infection. In this cell model, gal-FPd-BSA was also tested to be effective and nontoxic. The increase in cell surface expression of HBeAg precursor is meaningful since the less immune pressure due to the lack of HBeAg precursor expression is one of the most important mechanisms of persistent existence of HBeAg-defective HBV infection [15]. Side-effects are also key obstacles for the clinical usage of potential drugs. The hypotoxic of gal-FPd-BSA may be helpful. In addition, the enhanced efficiency did not influence the protein synthesizes of hepatocytes, concordant with furin not to be involved in proalbumin maturation [32]. Thus, these findings suggest that it is possible to inhibit furin via ASGP-R. However, our improvement only increased the inhibitory efficiency about 5 times, in other words, decreased the effective dosage from 100 μmol/L to 20 μmol/L, implying that there is a lot of space for further improvement when compared with the \textit{in vitro} \( K_i \) values (40-100 nmol/L) of the peptide inhibitors. As for gal-FPd-BSA, more further studies are imperative, especially the animal tests to examine the hepatocyte-targeting ability, the inhibitory efficiency, the hepatotoxicity and systematic side-effects, including autoimmune responses.

6. Conclusion

As the key convertase of HBeAg maturation, furin is thought as a potential target to promote HBeAg seroconversion. Unfortunately, there are currently no inhibitors meeting clinical needs with respect to efficiency and side effects. In this study, furin inhibitory peptides delivered via ASGP-R were found to be effective and nontoxic. Though there remains much work to do, this study highlights a new way to achieve HBeAg seroconversion, or even to control the infection by restoration of antiviral immunity in the future.

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