A polypeptide D-TTK001 targeting HBx controls cccDNA against HBV

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1   **A polypeptide D-TTK001 targeting HBx controls cccDNA against HBV**

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23

1   **Abstract**

2   Chronic infection of HBV is a serious global health issue. However, the HBV covalently closed
3   circular DNA (cccDNA) cannot be completely cleaned in liver. Here, we engineer a novel
4   polypeptide, termed D-TTK001, targeting HBV X protein (HBx) against HBV cccDNA. D-
5   TTK001 is a D-type polypeptide, which has a high binding affinity to HBx. Interestingly, it is able
6   to against HBV efficiently in primary human hepatocytes (PHHs) from three donors. D-TTK001
7   displays high activities against HBV, remarkably reducing the levels of cccDNA in the HBV-
8   infected human liver-chimeric mice. Mechanically, D-TTK001 depresses cccDNA transcription
9   by regulating the epigenetic modification of cccDNA minichromosome and disturbing the
10   interaction of HBx with DDB1 in the cells. D-TTK001 decreases the levels of cccDNA by down-
11   regulating MSL2, leading to the increase of APOBEC3B in liver. When combined with entecavir
12   or interferon- α , D-TTK001 shows additive antiviral effects. Clinically, D-TTK001 is a strong
13   candidate to clear cccDNA against HBV.

1   **Introduction**

2   Hepatitis B virus (HBV) is a prototypical hepadnavirus that specifically infects hepatocytes ^{1,2}.
3   Persistent HBV infection stimulates the development of chronic hepatitis B (CHB), liver cirrhosis,
4   and hepatocellular carcinoma (HCC), which is a leading cause of liver cancer worldwide ^{2,3}.
5   However, the current therapy rarely leads to HBV cure, and lifelong therapy is often required,
6   contributing to the poor uptake and ongoing morbidity.

7   HBV covalently closed circular DNA (cccDNA) is the genomic form of HBV, playing crucial
8   roles in the viral transcription and viral persistence. The level of HBV replication is determined by
9   the number of cccDNA molecules and their transcriptional productivity ⁴. HBV cccDNA
10   transcription is regulated by epigenetic modifications, including DNA methylation, histone
11   modification, nucleosome remodeling, and RNA-mediated targeting ⁵⁻⁸, which are imposed by
12   viral and cellular proteins as well as inflammatory cytokines ⁹. HBV X protein (HBx) as a
13   multifunctional HBV protein is crucial for HBV replication, pathogenesis, and hepatocyte
14   carcinogenesis ¹⁰. HBx interacts with cccDNA and activates transcription ¹¹. Multiple evidence
15   from our team and others demonstrate that HBx is a key antiviral target ^{2,12-14}. Considering that the
16   potential clinical importance of chronic HBV infection and liver diseases, and the central role of
17   HBx in HBV replication, it is urgent to develop novel anti-HBV drugs targeting HBx protein to
18   cure HBV-infected diseases ¹⁰.

19   Currently, the recommended therapeutic reagents for chronic HBV infection include nucleotide
20   analogs (NAs) and interferon- α (IFN- α)/pegylated IFN- α . NAs inhibit the replication late in the
21   viral life cycle with limited or no effect on viral protein production or on the persistence of
22   cccDNA resulting in high rates of relapse once NAs are discontinued ^{15,16}. Research shows that
23   IFN- α induces the decay of established HBV cccDNA, but it is limited by its adverse effects and

1 low response rate around 20-40% of the whole IFN- α therapy¹⁷. Because the current anti-HBV
2 therapy is unsatisfactory, the new antiviral strategies and drugs are urgently needed.

3 Proteins carry out the majority of the biological processes, and it is generally accepted that
4 protein-protein interactions (PPIs) are responsible for the cellular physiological and pathological
5 processes¹⁸⁻²⁰. Consequently, some existing polypeptide drugs are developed according to the
6 therapeutic strategy of PPI inhibition²¹⁻²³. PPIs are critical for understanding virus-host
7 interactions, and the biological functions of HBx are dependent on the pleiotropic PPIs¹⁰.
8 However, the anti-HBV drugs have not been reported according to the therapeutic strategy of PPI
9 inhibition.

10 In this study, we engineered a novel polypeptide targeting HBx and evaluated its preclinical
11 anti-HBV efficacy *in vitro* and *in vivo*. Surprisingly, our data demonstrated that the engineered D-
12 type polypeptide derived from hepatitis B X-interacting protein (HBXIP) sequence remarkably
13 reduced the levels of cccDNA in primary human hepatocytes (PHHs) and HBV-infected human
14 liver-chimeric mice. Our finding provides a new strategy for controlling cccDNA against HBV.

15

16 **Results**

17 **A novel engineered D-type polypeptide**

18 According to the drug deployment strategy through PPIs, we tried to identify the polypeptides
19 targeting HBx, leading to alter its biologic properties, from the candidate proteins binding to HBx.
20 Interestingly, HBXIP, which negatively regulated HBx activity and controlled the replication life
21 cycle of HBV²⁴, stood out from the candidates. Moreover, we designed and screened several
22 fragments from HBXIP, and finally, a fragment containing 12 amino acids (aa) termed TTK001
23 (25-36 aa in HBXIP) was selected (Fig.1a). We changed all L-type amino acids of TTK001 (or

1 TK) into D-type amino acids, termed D-TTK001 (or D-TK). Then, we viewed the interaction
2 between TTK001/D-TTK001 and HBx using an in silico model, which was performed by using
3 BIOVIA Discovery Studio 2016 (DS2016). Both TTK001 and D-TTK001 were compatible with
4 the orthostatic amino acid binding pocket of HBx, and the mutants of both significantly changed
5 the interaction of polypeptide with HBx (Fig.S1a,b). Furthermore, surface plasmon resonance
6 (SPR) showed that the dissociation constant (KD) value of TTK001/D-TTK001 was 15.06 nM and
7 14.09 nM (Fig. 1b, c). GST pull-down and co-immunoprecipitation (Co-IP) assays verified that
8 HBx was able to interact with TTK001 (Fig.1d, e). As expected, D-TTK001 could block the
9 interaction of endogenous or exogenous HBx with HBXIP (Fig.1f, g, Fig.S1c-e). To increase the
10 half-life of the polypeptide, we selected D-type TTK001 (D-TTK001) for subsequent
11 development. We further evaluated the ability of D-TTK001 to enter the host cells. Surprisingly,
12 we observed the red fluorescence during the time point of 5 min to 90 min after incubated with red
13 luciferin cy3-labeled D-TTK001 (100 μ M) in HepG2.2.15 and HepAD38 (Tet off) cells by
14 immunofluorescence assays (Fig.1h, i), suggesting that D-TTK001 is able to enter the cells with
15 high membrane permeability. Taken together, we conclude that D-TTK001 is a new D-type
16 polypeptide derived from the HBXIP sequence with a high binding affinity to HBx.

17 To evaluate the stability of D-TTK001 in animals, the preclinical metabolism and
18 pharmacokinetic studies of D-TTK001 were conducted (Fig.S1f). Our data showed that D-
19 TTK001 had a half-life of approximately 0.33-0.67 h in rats and 0.840-1.61 h in the monkeys (Fig.
20 S1g-j). After subcutaneous injection of D-TTK001, the main parameters of the D-TTK001 plasma
21 exposure, Cmax and AUC, increased proportionally with the increasing dose, and D-TTK001 had
22 no obvious accumulation trend (Table S1-S4).

23 **Efficacy of D-TTK001 against HBV *in vitro***

We evaluated the anti-HBV efficacy of D-TTK001 *in vitro*. The cell lines used for studying HBV included HepAD38 cell line which replicated HBV under the regulation of tetracycline (Tet) that supporting steps in replication, dHepaRG and HepG2-NTCP cell lines that supporting HBV infection, and primary human hepatocytes (PHHs) that supporting complete HBV life cycle ²⁵. Firstly, in HepAD38 (Tet off) cell lines, we confirmed the anti-HBV efficacy of D-TTK001 with high concentration (100 μ M). Compared with the control group, D-TTK001 reduced the levels of HBV DNA, HBsAg, HBeAg, and HBcAg, and MTS assays showed that D-TTK001 had no chemical toxicity in this concentration (Fig.S2a). Then, we determined the appropriate drug action concentration used for further research. The results showed that D-TTK001 had anti-HBV efficacy in a dose-dependent manner, and D-TTK001 concentration had no cytotoxicity below 200 μ M (Fig.S2b). Then, we further evaluated the anti-HBV efficacy of D-TTK001 in different cells. D-TTK001 decreased the levels of HBV, HBsAg, HBeAg, and HBcAg without affecting the viability of cells (Fig.2a-j). D-TTK001 displayed the antiviral effect, with around EC₅₀ of 7.21-13.8 μ M in PHHs, HepG2-NTCP, HepAD38 (Tet off), and dHepaRG cell lines (Fig.2a, f, Fig.S2c-f). ETV and IFN- α were selected as positive control drugs (Fig.2e, j). Hence, we conclude that D-TTK001 is able to against HBV *in vitro*.

Efficacy of D-TTK001 against HBV *in vivo*

We next evaluated the anti-HBV efficacy of D-TTK001 *in vivo*. The HBV-infected human liver-chimeric mouse (HBV-Huhep-URG mice) was used to evaluate the anti-HBV efficacy of D-TTK001 (Fig.3a). The qPCR assays showed that the levels of HBV DNA were above 1.00×10^5 IU/mL in the serum (Table S5), and IHC staining displayed that human albumin (hALB), HBsAg and HBcAg were positive in the liver tissue samples (Fig.S3a), suggesting that HBV-infected human liver-chimeric mouse model was successfully established. Statistical analysis showed no

1 significant difference in body weight change among mice of different groups (Fig.S3b). Our data
2 showed that the levels of HBV DNA were decreased in serum from the D-TTK001 treated mice
3 one week later after the treatment, with a final decrease of 2.2 (2.5 mg/kg group), 2.5 (5 mg/kg
4 group) and 2.8 (10 mg/kg group) log value (HBV IU/mL) in four weeks (Fig.3b, c). The levels of
5 HBeAg and HBsAg were significantly reduced in the serum from experiment groups compared
6 with control group (Fig.S3c, d). IHC staining revealed that the levels of HBsAg and HBcAg were
7 markedly reduced in the liver tissues from the mice treated with D-TTK001 in comparison with
8 control group (Fig.3d). RT-qPCR showed that the levels of HBV mRNA fragments, such as
9 HBx/pgRNA, HBs mRNA, and HBc/HBe mRNA, were obviously decreased in the liver tissues
10 treated by the D-TTK001, relative to 5% Glu group (Fig.S3e-g). Moreover, we observed that the
11 morphological character was improved in the liver tissues from the mice in the system (Fig.3d).
12 Interestingly, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)
13 were significantly decreased in the serum from D-TTK001-treated mice (Fig.3e, f). ETV was used
14 as a positive control in the system (Fig.3b-f, Fig.S3c-g). We also assessed the efficacy of D-
15 TTK001 against HBV using hydrodynamic transfection mouse model (Fig.S3h). The weight of the
16 experiment mice was showed in Fig.S3i. As expected, the levels of HBV DNA on day 21 and Day
17 28 were significantly dropped compared with Day 0 in the serum from the mice treated with D-
18 TTK001 (Fig.S3j). Taken together, we conclude that D-TTK001 is able to against HBV in animal
19 models.

20 We characterized the *in vivo* toxicity of D-TTK001 that may be useful for its further clinical
21 trial. A straightforward approach to test the acute toxicity would be to challenge the animal with
22 high doses of the drug. Therefore, we tested the acute toxicity in mice by challenging with D-
23 TTK001 *via* tail vein at 1000 mg/kg (Fig.S4a). The gross appearance, body weight, the hemogram

1 index, and histopathology of major organs showed no toxic changes after the treatment with D-
2 TTK001 (Fig.S4b, c, Table S6), suggesting that D-TTK001 with 1000 mg/kg high doses displays
3 no toxicity in mice. We also performed long-term toxicity experiment to test the *in vivo* toxicity of
4 D-TTK001. The rats were given subcutaneous injection of D-TTK001 at 40 mg/kg once a day for
5 22 days (Fig.S4d). There was no significant influence of D-TTK001 on the gross appearance,
6 body weight, and pathological observation (Fig.S4e, f). Although some hematology index and
7 serum biochemical index were changed, the extent was moderate (Table S7-8). However, the
8 blood electrolyte, organ weight and organ coefficients of the rats in different groups showed no
9 obvious difference (Table S9-11). Thus, we conclude that D-TTK001 is safety in mice and rats.

10 **D-TTK001 hijacks HBV cccDNA in liver**

11 Given that HBV cccDNA was notoriously refractory to the NAs commonly used in clinical
12 practice²⁶, we were interested in whether D-TTK001 was available to depress HBV cccDNA.
13 Strikingly, qPCR showed that D-TTK001 remarkably decreased the levels of cccDNA in a dose-
14 dependent manner in PHHs, HepAD38, dHepaRG and HepG2-NTCP cell lines (Fig.4a, Fig.S5a-
15 d). Moreover, Southern blot analysis demonstrated that D-TTK001 dramatically reduced the levels
16 of cccDNA in the system (Fig.4b, c), suggesting that D-TTK001 is able to reduce the cccDNA
17 levels in hepatocytes. ETV and IFN- α were served as positive controls (Fig.4a, b, Fig.S5a-d). As
18 expected, qPCR and Southern blot analysis displayed that D-TTK001 decreased the levels of
19 cccDNA in a dose-dependent manner (2.5, 5 and 10 mg/kg) in the liver from HBV-infected human
20 liver-chimeric mice (Fig.4d, e). HBV cccDNA samples of D-TTK001 2.5 mg/kg group and
21 control group were digested with EcoRI (Fig.4e, right lane). However, ETV failed to work in the
22 system (Fig.4e), only reducing the levels of relaxed circular DNA (rcDNA) and double-stranded

1 linear DNA (dsDNA). Thus, we conclude that D-TTK001 efficiently controls the levels of HBV
2 cccDNA *in vitro* and *in vivo*.

3 **The mechanisms of D-TTK001 against HBV**

4 Next, we sought to explore the underlying mechanisms by which D-TTK001 controlled HBV
5 cccDNA and inhibited HBV replication. To provide the evidence that D-TTK001 worked in the
6 cells, we demonstrated the localization of D-TTK001 and HBx by immunofluorescence (IF)
7 confocal microscopy. Interestingly, we observed that most of HBx (green) were limited in the
8 cytoplasm of HepAD38 (Tet off) cells from the situation of diffuse distribution after D-TTK001
9 (red) treatment (Fig.5a). Moreover, Western blot analysis verified that the expression levels of
10 HBx were decreased in a dose-dependent manner in the nucleus after D-TTK001 treatment
11 (Fig.5b, c, Fig.S6a), suggesting that D-TTK001 blocks the nucleus entry of HBx.

12 Considering that nuclear HBx bound to the HBV minichromosome and modified the epigenetic
13 regulation of cccDNA ⁷, we further investigated the effect of D-TTK001 on the epigenetic
14 modification of HBV cccDNA. Co-IP assay showed that D-TTK001 treatment attenuated the
15 binding of HBx to p300 (Fig.S6b, S6c). ChIP assays revealed that D-TTK001 treatment
16 significantly decreased the levels of AcH3, H3K4me3, EP300, and CBP, and promoted the
17 deposition of H3K9me3, H3K27me3, HDAC1, SIRT1, and EZH2 on the cccDNA
18 minichromosome (Fig.5d, e). Thereby, we summarize that D-TTK001 affects epigenetic
19 modification of cccDNA minichromosome due to blocking the nuclear entry of HBx (Fig.S6d),
20 leading to the repression of transcription of HBV DNA. Given that HBx was able to enhance the
21 HBV transcription by hijacking DDB1-containing E3 ubiquitin ligase to target the Smc5/6 for
22 proteasomal degradation ²⁷, we performed Co-IP assays to identify whether D-TTK001 disturbed
23 the interaction of HBx with DDB1 in the cells. As expected, D-TTK001 prevented the binding of

1 endogenous and exogenous HBx with DDB1 in HEK293T, HepG2, and HepG2-NTCP cell lines
2 (Fig.5f, g, Fig.S6e). Next, we tested whether D-TTK001 may prevent Smc5/6 degradation induced
3 by HBV. Consistently, D-TTK001 strongly increased the levels of Smc5/6 in HepAD38 (Tet off)
4 and HepG2-NTCP cell lines (Fig.5h, Fig.S6f). Thus, we summarize that D-TTK001 blocks the
5 interaction of HBx with DDB1, leading to the prevention of Smc5/6 degradation (Fig.S6g).

6 It had been reported that the cytosine deaminization activity of APOBEC3A or APOBEC3B is
7 essential for inducing cccDNA degradation ¹⁷, and MSL2 mediated by HBx contributes to the
8 degradation of APOBEC3B through ubiquitylation ¹². Accordingly, our data showed that D-
9 TTK001 down-regulated the levels of MSL2 and increased the levels of APOBEC3B in HepAD38
10 (Tet off) and HepG2-NTCP cell lines (Fig.5g, f).

11 Interestingly, we further observed that D-TTK001-mut failed to work in repressing HBV
12 biomarkers, and the over-expression of HBx could remarkably neutralize the efficacy of D-
13 TTK001 in PHH, HepAD38 (Tet off) and HepG2-NTCP cell lines (Fig.5i, j, Fig.S6h), supporting
14 that D-TTK001 works through targeting HBx. Thus, we conclude that the mechanisms of D-
15 TTK001 against HBV include: depressing cccDNA transcription by regulating the epigenetic
16 modification of cccDNA minichromosome and disturbing the interaction of HBx with DDB1;
17 decreasing the levels of cccDNA by down-regulating MSL2 which leads to the increase of
18 APOBEC3B in liver.

19 **Combination of D-TTK001 with ETV or IFN- α**

20 Currently, the treatment guidelines recommend combination therapy for treating multidrug-
21 resistant CHB ²⁸. Accordingly, we were interested in whether D-TTK001 had synergistic anti-
22 HBV effects when combined with ETV or IFN- α . Compared with the single-drug group, D-
23 TTK001 and ETV coadministration reduced the levels of HBV DNA, HBsAg, and HBeAg in

1 HepAD38 cell line (Fig.6a-c, Fig.S7a-c). D-TTK001 and IFN- α coadministration displayed the
2 consistent effects (Fig.6E-G, Fig.S7D-F), suggesting that D-TTK001 has synergistic anti-HBV
3 effects with ETV or IFN- α . MTS assays showed that the combined treatment of D-TTK001 with
4 ETV or IFN- α had no additional toxicity (Fig.6d, 6h). Collectively, we conclude that the
5 combination of D-TTK001 with ETV or IFN- α displays synergistic anti-HBV effects *in vitro*.

6

7 **Discussion**

8 Chronic hepatitis B, caused by HBV infection, is a challenging prevalent infectious disease ²⁹.
9 Importantly, HBV cccDNA is an obstacle in the treatment of chronic HBV infection ⁴. However,
10 the current therapy rarely leads to cccDNA elimination. Thus, a new generation of anti-HBV
11 drugs that successfully block cccDNA is urgently needed. Here, we try to engineer a novel
12 polypeptide targeting HBx to clear cccDNA.

13 HBx, as a multifunctional exogenous virus protein, is an important therapeutic target for the
14 interruption of chronic HBV replication ^{10,30}. However, the anti-HBV drugs targeting HBx protein
15 have not been reported yet. The biological functions of HBx are mainly dependent on pleiotropic
16 PPIs ^{27,31-33}. According to the drug deployment strategy through PPIs, we screened a series of
17 interacting proteins, and finally identified a fragment from HBXIP sequence, termed TTK001. As
18 expected, TTK001 could block the interaction of endogenous or exogenous HBx with HBXIP. We
19 changed all L-type amino acids of TTK001 into D-type amino acids, termed D-TTK001.
20 Interestingly, we demonstrated the phenotype of the interaction of HBx with the polypeptide in 3D
21 structure using in silico model, but not the crystal model due to the crystal full structure of HBx
22 was unavailable. Moreover, our data validated that D-TTK001 KD value was 14.09 nM. It
23 suggested that D-TTK001 strongly binds to HBx protein. Because the polypeptide in clinic is

1 limited by their susceptibility to enzymatic degradation, to improve the half-life of the TTK001,
2 we selected D-type TTK001 (D-TTK001) for subsequent development. Strikingly, the red
3 fluorescence that labeled D-TTK001 was observed in the cells. It implies that D-TTK001 can
4 work in the cells. The pharmacokinetic studies showed that D-TTK001 had a half-life of
5 approximately 0.33-0.67 h in rats and 0.840-1.61 h in monkeys. It strongly supports that D-
6 TTK001 is available against HBV in humans.

7 Next, we evaluated the efficacy of D-TTK001 against HBV *in vitro* and *in vivo*. The human
8 liver-chimeric mouse model was developed by partially repopulating the mouse liver with primary
9 human hepatocytes, and HBV was able to infect and replicate³⁴, it is an ideal model that offers a
10 unique opportunity to evaluate the *in vivo* efficacy of antiviral compounds targeting different steps
11 of the HBV life-cycle^{35,36}, and especially it allows to test cccDNA in the liver³⁷. As expected, our
12 data demonstrated that D-TTK001 was able to significantly depress the viral biomarkers in the cell
13 lines and animal models. Ideally, a cure for HBV infection needs to eliminate cccDNA, thereby
14 preventing future reactivation. Accordingly, we focused on the investigation of cccDNA in this
15 study. Notably, qPCR and Southern blot analysis revealed that D-TTK001 remarkably decreased
16 the levels of cccDNA. In this study, ETV and IFN- α worked well as positive controls. However,
17 ETV failed to control cccDNA. Our D-TTK001 is better than ETV in controlling cccDNA.
18 Besides, we demonstrated that D-TTK001 was safe in mice and rats by using acute toxicity and
19 long-term toxicity experiments. We concerned whether D-TTK001 generated the antibody in
20 serum for long time treatment. However, the antibody against D-TTK001 could not be detected in
21 monkeys with exposure of D-TTK001 (data not shown). It strongly suggests that D-TTK001 is
22 available in clinic controls cccDNA against HBV.

Mechanically, we investigated the details of D-TTK001 against HBV. We validated that D-TTK001 worked through interacting with HBx in the cells. Interestingly, D-TTK001 inhibited the nuclear translocation of HBx in the cells. Nuclear HBx protein stimulates virus replication *via* histone modifications on cccDNA³⁸. Interestingly, we found that D-TTK001 modulated the epigenetic regulation of cccDNA. Importantly, it has been reported that Smc5/6 binds to cccDNA minichromosome to block its transcription, and HBx binds to the DDB1 to target Smc5/6 complex for ubiquitin-mediated degradation²⁷. As expected, our finding showed that D-TTK001 repressed the interaction of HBx with DDB1, increasing Smc5/6 protein levels. It had been reported that APOBEC3A and APOBEC3B are responsible for the deamination of HBV cccDNA¹⁷. Our previous study showed that MSL2 could mediate the ubiquitylation of APOBEC3B and HBx up-regulated MSL2 to activate HBV cccDNA, forming a positive feedback loop of HBx/MSL2/cccDNA/HBV¹². Consistently, in this study we demonstrated that D-TTK001 reduced the levels of MSL2 and elevated APOBEC3B expression in the cells. However, it is elusive for the targets of D-TTK001 besides HBx in the cells.

Next, we moved to the combination of drugs. Numerous studies demonstrated superior results with combination therapy than monotherapy alone, and HBV cure needs a combination of new targets drugs and antiviral strategies^{39,40}. In this study, our finding revealed that the combination of D-TTK001 with ETV or IFN- α increased the efficacy against HBV *in vitro*. It suggests that the combination of drugs with different targets works well and will be significant in clinic.

Taken together, we summarize a model that D-TTK001 controls cccDNA against HBV (Fig.S8). TTK001 is identified from the fragment of HBXIP sequence by PPI strategy. All of the amino acids of TTK001 are reversed into D-type amino acids, termed D-TTK001. Mechanically,

1 D-TTK001 depresses cccDNA transcription through regulating the epigenetic modification of
2 cccDNA minichromosome and disturbing the interaction of HBx with DDB1; and decreases the
3 levels of cccDNA by down-regulating MSL2 which leads to the increase of APOBEC3B in liver.

4 5 **Methods**

6 **Animals**

7 The HBV-infected human liver-chimeric mice (HBV-Hu hep-URG mice) were generated by
8 Beijing Vitalstar Biotechnology Co. Ltd.. (Beijing, China). Serum HBV load in the mice was
9 determined by quantitative PCR (Da An Gene, Guangzhou, China) before sacrifice. The
10 information about HBV-infected human liver-chimeric mice was shown in Table S5. Stock
11 solutions of ETV (Sigma-Aldrich, USA) in PBS was further diluted in 5% Glucose before
12 intragastric administration (i.g.). D-TTK001 was generated by Shanghai AmbioPharm, Inc and
13 was diluted with 5% Glucose before subcutaneous (s.c.) injection. The mice were divided into 6
14 groups, including D-TTK001-10 mg/kg, D-TTK001-5 mg/kg, D-TTK001-2.5 mg/kg groups, ETV
15 (0.076 mg/kg) positive control group, and vehicle (5% glucose injection) negative control group, 6
16 mice each group. The serum samples were collected from all groups of mice at the time point of
17 pre-dose (Day 0), 1 week (Day 7), 2 weeks (Day 14), 3 weeks (Day 21), and 4 weeks (Day 28)
18 after treatment. After daily administration for 28 days, mice were sacrificed and livers of mice
19 were collected for quantitative polymerase chain reaction (qPCR) analysis, pathological
20 observation, and immunostaining. The pathological observation was assessed by two independent
21 pathologists with no prior knowledge of the sample characteristics, which was completed by
22 Beijing Dian Diagnostics Laboratory Co., Ltd.. Discrepancies were resolved by consensus. All the
23 analyses were performed blindly.

The hydrodynamic injection (HDI)-based HBV-carrier models were constructed as previously described by using pAAV-HBV1.2 plasmid. The mice were divided into 3 groups, including D-TTK001-10 mg/kg group, ETV (0.076 mg/kg) positive control group and vehicle (5% glucose injection) negative control group, 6 mice each group. The serum samples were collected from all groups of mice at the time point of pre-dose (Day 0), 1 week (Day 7), 2 weeks (Day14), 3 weeks (Day 21), and 4 weeks (Day 28) after treatment. After daily administration for 28 days, mice were sacrificed.

Sprague–Dawley rats (3 months, weighting 200-250 g for female, 300-360 g for male) and cynomolgus monkeys (5 years old, weighting 2.6-3.8 kg) with half males and half females were used to test the pharmacokinetic study of D-TTK001 (as shown in Fig.S1F). Pharmacokinetic studies of D-TTK001 were completed by Tianjin Taincheng New Drug Evaluation Co. Ltd.. For single subcutaneous (SC) injection tests, rats and monkeys were separated into 3 groups (1, 2, 4 mg/kg D-TTK001 for rats, and 0.5, 1, 2 mg/kg D-TTK001 for monkeys), 6 animals in each group, a single dose only. For multiple subcutaneous injection tests, rats and monkeys were exposed to continuous subcutaneous administration of D-TTK001 once daily for 7 days (2 mg/kg for rats, and 1 mg/kg for monkeys), 6 animals in each group. After SC administration, 200 μ L of blood was collected at different specific time points (0.0833, 0.167, 0.25, 0.5, 0.75, 1.5, 2, 3, 4, 5, and 6 h for rats, and 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h for monkeys) and frozen for pharmacokinetics studies. D-TTK001 concentration and pharmacokinetic parameters were then determined by liquid chromatography and mass spectroscopy (API5000, Applied Biosystems).

Sprague-Dawley rats (2 months, weighting 190 ± 5 g for female, 220 ± 10 g for male) and Swiss albino mice (6 weeks of age, weighting 25 ± 1 g for female, 29 ± 1 g for male) with half males and half females were used for drug toxicity assays (as shown in Fig.S4A, S4D). The toxicity

1 studies of D-TTK001 were completed by Tianjin Taincheng New Drug Evaluation Co. Ltd.. For
2 acute toxicity testing, the mice were given a single dose of 1000 mg/kg of D-TTK001. The mice
3 were observed once daily and weighed every 2 days for 14 days. The blood sample of 100 μ L was
4 collected at day 7 and day 14 for hemogram determined. Mice were euthanized at the endpoint,
5 and haematoxylin and eosin (H&E) staining in different organs/tissues was performed. For long-
6 term toxicity experiment, rats were continuous subcutaneous administration of D-TTK001 once
7 daily for 7 days (40 mg/kg). Rats were observed once daily and weighed at day 1,4,13 and 21. At
8 the endpoint of the experiment, the rats were euthanized. The blood samples were collected for
9 blood routine, coagulation function, biochemical and electrolyte test, and the organs were
10 harvested for organ ratios and pathology determining.

11 The experiments were conducted following the Guide for the Care and Use of Laboratory
12 Animals. The Institute Research Ethics Committee at Nankai University approved the study
13 protocol.

14 **Cell lines**

15 HEK293T, HepG2, HepG2-NTCP, and HepG2.2.15 cells were cultured in DMEM medium
16 (Invitrogen, Carlsbad, California, USA) supplemented with 10% (vol/vol) fetal bovine serum
17 (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μ g /mL streptomycin. dHepaRG cell lines
18 were cultured in RPMI Medium 1640 (Invitrogen, Carlsbad, California, USA) supplemented with
19 10% (vol/vol) fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL).

20 The HepAD38 cell line regulates HBV replication through the presence or absence of tetracycline
21 in the culture medium. HepAD38 cell was cultured in DMEM/F12 medium (Life Technologies,
22 Carlsbad, CA) supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, 100 μ g/mL
23 streptomycin, 100 μ g/mL kanamycin, 400 μ g/mL G418, and with 1 μ g/mL tetracycline (for

inhibition of HBV replication) or without any tetracycline (for induction of HBV replication). The primary human hepatocytes (PHH) of three donors were purchased from Shanghai RILD Inc. (Shanghai, China). The cells were cultured similarly using the same plating and incubation medium as described above³⁰. All the cell lines were cultured in 5% CO₂ at 37 °C.

HEK293T, HepG2, HepAD38, HepG2.2.15, HepG2-NTCP, and dHepaRG cells were transfected using VigoFect (Vigorous Biotechnology) or Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. For the treatment of D-TTK001, ETV or IFN- α stock solution was diluted to the final concentrations in the DMEM medium. HepG2-NTCP, dHepaRG cells, and PHHs were cultured and infected with HBV as described^{41,42}. The cells were washed with PBS three times and then cultured with D-TTK001, ETV or IFN- α for 7 days. The cytotoxicity was analyzed by CellTiter 96_AQueous One Solution Cell Proliferation Assay (G3580, Promega, USA) according to the manufacture's instruction.

Viruses

HBV particles were collected and purified from the supernatant of HepAD38 cells (Tet-off) as described³⁰. Media were recovered every 3 days from HepAD38 cells at day's 7-15 post-induction of HBV by depletion of tetracycline. The media were cleared through a 0.45 μ m filter and precipitated with 10% PEG8000 and 2.3% NaCl. The precipitates were washed and resuspended with the medium at 200-fold concentration. HBV DNA was quantified by qPCR. Only HBV particles reaching a titer between 3×10^9 and 3×10^{10} vp/mL were used. Immediately after collection, the virus stock was aliquoted and stored at -80 °C until used for infection. HepG2-NTCP, dHepaRG cells and PHH were infected with HBV particles at 500-1000 genome equivalents (GEq)/cell in the presence of 4% PEG8000 at 37 °C for 16 h as previously described^{43,44}.

1   **RNA extraction, reverse-transcription polymerase chain reaction (RT-PCR), and**
2   **quantitative real-time PCR (RT-qPCR)**

3   Total RNA was extracted from cells (or liver tissues from mice and patient tissues) using Trizol
4   reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand
5   cDNA was synthesized as reported before ⁴⁵. RT-qPCR was performed by a 7900 HT qRT-PCR
6   system (Applied Biosystems, Life Technologies Darmstadt, Germany) according to the
7   manufacturer's instructions using double-stranded DNA-specific SYBR Green Mix (TaKaRa,
8   Ohtsu, Japan). Experiments were conducted in three independent assays. Relative transcriptional
9   folds were calculated as $2^{-\Delta\Delta C_t}$. Relative mRNA levels of all target genes were normalized to at
10   least two house-keeping genes (HPRT; RHOT2; TBP; GAPDH) levels. All the primers used are
11   listed in Table S12.

12   **HBV cccDNA extraction, qPCR and Southern blot analysis**

13   Before lysis, cell number was counted by using Cell Counting Chamber Set (Qiujiang, Shanghai,
14   China). The procedure of the isolation and quantification of HBV cccDNA from HepAD38,
15   HepG2.2.15 cells or HBV infected PHH, dHepaRG, HepG2-NTCP cells and mice liver tissues
16   was performed according to previous research ⁴⁶⁻⁴⁸. qPCR experiments were performed in a qPCR
17   system (Eppendorf, Germany) using a 20 μ L reaction volume containing 2 μ L digested HBV DNA.
18   The primers used to amplify the cccDNA were listed in Supplementary KEY RESOURCES
19   TABLES. For Southern blot analysis, the extracted HBV cccDNA sample was separated through
20   1.0% agarose gel, blotted onto a nylon membrane, and hybridized with DIG-labeled probes of
21   linear HBx DNA fragments. When indicated, isolated DNA was digested with EcoRI that
22   linearizes the HBV cccDNA.

23   **Surface plasmon resonance (SPR)**

1 SPR experiments were carried out to characterize the binding of HBx protein to two peptides (D-
2 TTK001 and TTK001) using a Biacore T200 (GE Healthcare), which was completed by Shanghai
3 Ruizhi Chemistry Co., Ltd.. All SPR-based materials were purchased from GE Healthcare. D-
4 TTK001/TTK001 were diluted in HBS-EP and immobilized on CM5 sensor surface using amine
5 coupling chemistry. Biotin-polypeptides were diluted in HBS-EP and immobilized on an SA chip.
6 Interaction analyses were tested using HBS-EP as a running buffer. Increasing concentrations of
7 His-HBx (3.125, 6.25, 12.5, 25, 50, 100 nM) were injected using the ‘Kinetics/Affinity’ program.
8 A flow cell without immobilized peptide served as a non-specific binding control. K_a , K_d , and K_D
9 were determined using the ‘Kinetics’ model in the Biacore T200 evaluation software version 2.0.

10 **Western blot analysis**

11 Total protein lysates were extracted from hepatoma cells with RIPA buffer. Cytoplasmic and
12 nuclear fractions were prepared from 5×10^8 hepatoma cells by using Minute Cytoplasmic &
13 Nuclear Extraction Kits (SC-003, Invent Biotechnologies, Inc, Plymouth, MN, USA) according to
14 the manufacturer’s instructions. Protein concentrations were measured using the BCA Protein
15 Quantification Kit (YEASEN, China), and 20-50 μ g protein extracts were subjected to SDS-
16 PAGE. Then proteins were transferred to a PVDF membrane, blocked with 5% non-fat milk and
17 incubated with first antibodies for 16h at 4 °C. After incubation with secondary antibody against
18 mouse (1:10,000) or rabbit (1: 10,000) for 1h at 37 °C, the membrane was visualized by ECL
19 Western Blotting Detection Kit (GE Healthcare, Waukesha, WI, USA). The primary antibodies
20 used for Western blot analysis were listed in Table S13.

21 **Hepatitis B surface antigen (HBsAg) and Hepatitis B e antigen (HBeAg) quantification**

22 The levels of HBsAg in the supernatants of cultured cells was measured by diagnostic kit for
23 hepatitis B virus surface antigen according to the manufacturer’s instructions (Kehua Bio-

1 engineering, Shanghai, China). The cut-off value (COV) for HBsAg analysis was indicated as:
2 $COV = OD \text{ (negative control, NC)} / 0.100$. HBeAg in the supernatants of cultured cells was
3 measured by a diagnostic kit for Hepatitis B e antigen according to the manufacturer's instructions
4 (Kehua Bio-engineering, Shanghai, China). The cut-off value for HBeAg analysis was indicated
5 as: $COV = OD_{NC} \times 2.1$ ($0 < OD_{NC} \leq 0.050$, $COV = 0.050 \times 2.1 = 0.105$; $0.050 < OD_{NC} \leq 0.100$;
6 $OD_{NC} > 0.100$, invalidation). The levels of HBsAg and HBeAg in the serum of HBV-Hu hep-URG
7 mice were completed by Beijing Dian Diagnostics Laboratory Co., Ltd. using
8 electrochemiluminescence immunoassay (ECLIA) according to the manufacturer's instructions
9 (Roche, Mannheim, Germany).

10 **HBV DNA analysis**

11 HBV DNA was extracted in the supernatants of HepAD38 cells, HepG2.2.15 cells, and PHH,
12 HepG2-NTCP and dHepaRG cells infected with different doses of HBV particles using the Blood
13 & Cell Culture DNA kit (QIAGEN, Germany) following the manufacturer's instructions. The
14 qPCR was used to quantify HBV DNA copies according to a diagnostic kit for the quantification
15 of HBV DNA (Da An Gene, Guangzhou, China) in a Bio-Rad sequence detection system.

16 **Immunofluorescence analysis**

17 Cells were grown on acid-treated glass coverslips in 24-well plates. Treated cells were fixed with
18 ice-cold 4% paraformaldehyde for 10 min, washed three times with phosphate-buffered saline
19 (PBS), and permeabilized with 0.1% Triton X-100 in PBS for 30 min. After washing three times
20 with PBS, samples were blocked with 5% BSA for 2 h. Cells were incubated with primary
21 antibodies overnight at 4 °C. After being washed, the cells were incubated with fluorophore-
22 conjugated secondary antibody (DAKO, Denmark) and DAPI. After washing with PBS, slides

1 were mounted with glycerol and observed under an upright fluorescence microscope (Zeiss Axio
2 Imager Z1).

3 **GST-Pull-down**

4 The complementary DNA fragments encoding the TTK001 polypeptide and HBx were cloned into
5 pGEX-6p-1 and PET-28a vector, respectively. The products were transformed into Escherichia
6 coli BL21 competent cells to produce a GST-tagged TTK001 and His-tagged HBx fusion protein.
7 The GST-tagged TTK001 was purified using Glutathione Sepharose 4B beads (GE Healthcare)
8 according to the manufacturer's instructions. His-tagged HBx was purified according to
9 previously described ⁴⁹. GST-tagged TTK001 and His-tagged HBx were added to the resin and
10 incubated overnight at 4 °C. The resin was then washed five times with a wash solution, and the
11 proteins were eluted with glutathione elution buffer. The eluted proteins were detected by Western
12 blotting.

13 **Co-immunoprecipitation (Co-IP)**

14 The whole cellular extracts were prepared using IP lysis buffer (Beyotime) supplemented with a
15 complete protease inhibitor cocktail according to the manufacturer's instructions. Cell lysates
16 were then incubated with the IP antibody overnight at 4 °C on a rotating wheel. Then the cell
17 lysates were incubated with Protein A/G Magnetic beads (Millipore) for 4 h at 4 °C on a rotating
18 wheel. The protein-protein interaction was analyzed by immunoblotting.

19 **Chromatin immunoprecipitation (ChIP)**

20 ChIP assays were performed in HepAD38, HepG2-NTCP cells (treated with tet on and tet off)
21 according to the manufacturer's protocol (Epigentek Group Inc, Brooklyn, NY, USA) as
22 previously reported ⁵⁰. Immunoprecipitated DNA was quantified by qPCR using cccDNA specific
23 primers (Table S12). Samples were normalized to input DNA using the Δ Ct method and

1 calculated as percentage of the input. Results are expressed as the average of at least three
2 independent experiments. Error bars represent means $\pm$ SD (n=3). Statistical differences were
3 analyzed by Student's *t* test.

4 **Hematoxylin-eosin (H&E) staining and Immunohistochemistry (IHC)**

5 For IHC, animals were sacrificed and liver tissue samples was collected. Samples were fixed in
6 10% formalin for 24 h then embedded with paraffin, sectioned at 5 μ m onto slides. H&E staining
7 was carried out using an H&E staining kit (Beyotime, China) according to the manufacturer's
8 instruction. Immunohistochemical staining of samples was performed as previously reported⁵¹
9 and the according to primary antibody were used (Table S13). The samples were monitored
10 microscopically.

11 **Quantification and statistical analysis**

12 Each experiment was repeated at least three times. All analyses and graphs were generated with
13 GraphPad Prism 6. Statistical significance was assessed by comparing mean values ($\pm$ SD) using a
14 Student's *t* test and was assumed for *p<0.05; **p<0.01; ***p<0.001.

15

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3   C.L.S., X.Z.G., X.M.W., M.Z., M.Y., and Z.C.M.; Investigation, H.F.Y., X.D.Z., S.Z., S.Q.Z., and
4   L.N.Z.; Validation, H.L.Y., Y.F.W., J.P.W., W.Z., Y.G., and Y.Y.; Writing-Review & Editing,
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7

8   **Competing interests**

9   Authors declare no competing interests.

Fig. 1. A new D-type polypeptide derived from HBXIP sequence is engineered. a, A model of strategy against HBV for TTK001. **b**, Affinity analysis of HBx binding to TTK001 using SPR. **c**, Affinity analysis of HBx binding to D-TTK001 using SPR. **d**, The interaction of HBx with D-TTK001 was tested by GST-Pull-down assays. **e**, Co-IP assays to test the binding of HBx and D-TTK001. HEK293T cells were transfected with plasmids Flag-HBx, and cells were incubated with

1 HA-tagged TTK001 polypeptide. The interaction of HBx with TTK001 was examined by Co-IP
2 and immunoblotting. **f**, The effect of D-TTK001 on the binding of HBXIP with exogenous HBx
3 was measured by Co-IP assays in HEK293T cells transfected with plasmids Flag-HBx and treated
4 with D-TTK001 (200 μ M) for 7 days. **g**, The effect of D-TTK001 on the binding of HBXIP with
5 endogenous HBx was measured by Co-IP assays in HBV *de novo* HepG2-NTCP cells treated with
6 D-TTK001 (200 μ M) for 7 days. **h-i**, Immunofluorescence analysis of D-TTK001 (red) in
7 HepG2.2.15 and HepAD38 (Tet off) cells. HepG2.2.15 (h) and HepAD38 (i) cells were treated
8 with red luciferin cy3-labeled D-TTK001 (100 μ M) and determined by immunofluorescence
9 assays at different time points. DAPI (blue) stained the nuclei. Scale bars, 10 μ m.

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Fig. 2. Efficacy of D-TTK001 against HBV *in vitro*. **a**, The levels of HBV DNA were measured by qPCR in the supernatant of HBV *de novo* PHH cells. **b**, PHH cell viability was measured by MTS assay. **c**, The levels of HBsAg and HBeAg were examined by ELISA in the supernatant of HBV *de novo* PHH cells. **d**, The levels of HBcAg were determined by immunofluorescence staining in HBV *de novo* PHH cells. Scale bars, 50 μ m. **e**, The levels of HBV DNA, HBsAg, and HBeAg were tested by qPCR and ELISA assays in the supernatant of HBV *de novo* PHH cells. **f**, The levels of HBV DNA were measured by qPCR in the supernatant of HBV *de novo* HepG2-NTCP cells. **g**, HepG2-NTCP cell viability was evaluated by MTS assays. **h**, The levels of HBsAg and HBeAg were examined by ELISA in the supernatant of HBV *de novo* HepG2-NTCP cells. **i**, The level of HBcAg was measured by immunofluorescence staining in HBV *de novo* HepG2-NTCP cells. Scale bars, 50 μ m. **j**, The levels of HBV DNA, HBsAg, and HBeAg were determined by qPCR and ELISA assays in the supernatant of HBV *de novo* HepG2-NTCP cells. Data are

1 represented as means $\pm$ SD (n=3). ns, no significant; * p <0.05; ** p <0.01; *** p <0.001 (Student's t
2 test).
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Fig. 3. Efficacy of D-TTK001 against HBV in HBV-infected human liver-chimeric mice. **a**, A model of HBV-infected human liver-chimeric mice (HBV-HuHep-URG mice). **b-c**, The level of HBV DNA was tested by qPCR in the serum of human liver-chimeric mice. The HBV-HuHep-URG mice were given dose-administration (2.5, 5, 10 mg/kg) once a day for 4 weeks. Serum samples were collected before exposure (Day 0) and 1, 2, 3, 4 weeks post-exposure (Day 7, Day 14, Day 21, Day 28), respectively. **d**, Pathological observation and immunohistochemistry staining for HBsAg and HBcAg were measured in the liver tissues from HBV-HuHep-URG mice with different treatments. Scale bars: 20 μ m. **e-f**, The levels of ALT and AST were evaluated by ALT/AST detection kits in the serum of HBV-HuHep-URG mice. The HBV-HuHep-URG mice were given dose-administration once a day for 4 weeks. Serum samples were collected after

1 exposure (Day 28). Data are represented as means $\pm$ SD (n=3). * p <0.05; ** p <0.01; *** p <0.001
2 (Student's t test).
3

Fig. 4. D-TTK001 efficiently controls the levels of HBV cccDNA *in vitro* and *in vivo*. **a**, The cccDNA levels were measured by qPCR assays in the nucleus of HBV *de novo* PHH cells treated with D-TTK001, ETV or IFN- α for 7 days. **b**, The cccDNA levels were examined by Southern blot analysis in the nucleus of HBV *de novo* PHH cells treated with D-TTK001 (200 μ M), ETV (100 nM), and IFN- α (2000 IU/mL) for 7 days. **c**, The cccDNA levels were measured by Southern blot analysis in the nucleus of cells of HepAD38 (Tet off), HBV *de novo* PHH, dHepaRG, and HepG2-NTCP treated with D-TTK001 (200 μ M) for 7 days. **d**, The cccDNA levels were determined by qPCR assays in the liver of HBV-Hu hep-URG mice treated with D-TTK001 (10, 5, 2.5 mg/kg) and ETV (0.076 mg/kg) for 28 days. **e**, The cccDNA levels were tested by Southern blot analysis in the liver of HBV-Hu hep-URG mice. Data are represented as means $\pm$ SD.

* p <0.05; ** p <0.01; *** p <0.001 (Student's t test).

Fig. 5. The mechanisms of D-TTK001 against HBV. **a**, Immunofluorescence staining was used to identify the location of D-TTK001 (red) and HBx (green) in HepAD38 (Tet off) and HepG2 cells treated with or without Red luciferin cy3-labeled D-TTK001 (100 μM). DAPI (blue) stained

1 the nuclei. Scale bars: 10 μ m. **b-c**, The effect of D-TTK001 on the expression of HBx was tested
2 by Western blot analysis in HepAD38 (Tet off) and HBV *de novo* PHH cells treated with D-
3 TTK001 for 7 days. **d-e**, The assembly of histone H3, AcH3, H3K4me3, H3K9me3, H3K27me3,
4 EP300, CBP, HDAC1, SIRT1, and EZH2 onto cccDNA was determined by ChIP-qPCR in
5 HepaAD38 (Tet off) or HBV *de novo* HepG2-NTCP cells treated with D-TTK001 for 7 days,
6 respectively. **f**, The interaction of DDB1 with exogenous HBx was examined by Co-IP assay in
7 HEK293T cells transfected with Flag-tagged HBx and treated with D-TTK001 for 7 days. **g**, The
8 effect of D-TTK001 on the expression of SMC5, SMC6, MSL2 and APOBEC3B was evaluated
9 by Western blot analysis in HBV *de novo* HepG2-NTCP cells treated with D-TTK001 for 7 days.
10 **h**, The effect of D-TTK001 on the expression of SMC5, SMC6, MSL2 and APOBEC3B was
11 evaluated by Western blot analysis in HepAD38 cells treated with D-TTK001 for 7 days. **i**, The
12 levels of HBV DNA, HBsAg, and HBeAg were measured by qPCR and ELISA assays in the
13 supernatant of HBV *de novo* PHH cells treated with D-TTK001 (200 μ M), D-TTK001-mut (200
14 μ M), ETV (100 nM) for 7 days. The pcDNA3.1-HBx (2 μ g) and pcDNA3.1-Vector (2 μ g) were
15 transfected at time points of day 0, day 2, and day 4. Data are represented as means $\pm$ SD (n=3).
16 * p <0.05; ** p <0.01; *** p <0.001 (Student's t test).

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Fig. 6. Efficacy of the combination of D-TTK001 with ETV or IFN- α *in vitro*. a-c, The levels of HBV DNA, HBsAg, and HBeAg were measured by qPCR and ELISA assays in the supernatant of HepAD38 (Tet off) cells treated with both D-TTK001 and ETV for 7 days. d, HepAD38 cell viability was tested by MTS assays. e-g, The levels of HBV DNA, HBsAg, and HBeAg were evaluated by qPCR and ELISA assays in the supernatant of HepAD38 (Tet off) cells treated with both D-TTK001 and IFN- α for 7 days. (H) HepAD38 cell viability was tested by MTS assays. Data are represented as means $\pm$ SD (n=3). * p <0.05; ** p <0.01; *** p <0.001 (Student's t test).

Fig. S1. A new D-type polypeptide derived from HBXIP sequence is engineered. **a**, A model of the interaction of TTK001/D-TTK001 (TK/D-TK) with HBx using in silico model. **b**, The structures of TTK001, D-TTK001, TTK001-mut and D-TTK001-mut were demonstrated by using in silico model. **c**, The effect of D-TTK001 on the binding of HBXIP with exogenous HBx was examined by Co-IP assays in HepG2 cells transfected with Flag-HBx and incubated with D-TTK001. **d-e**, The effect of D-TTK001 on the binding of HBXIP with endogenous HBx was

1 tested by Co-IP assays in HBV *de novo* HepG2-NTCP and HepAD38 (Tet off) cells treated with
2 D-TTK001 for 7 days. **f**, A model showed the protocol of the pharmacokinetic experiment. **g-j**,
3 Pharmacokinetics of indicated D-TTK001 doses in plasma after single or multiple subcutaneous
4 administration in rats and monkeys.

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Fig. S2. Efficacy of D-TTK001 against HBV *in vitro*. **a**, The levels of HBV DNA, HBsAg, HBeAg, HBcAg, and cell viability were respectively examined by RT-qPCR, ELISA, immunofluorescence and MTS assays in HepAD38 (Tet off) cells treated with D-TTK001 (100 μ M) for 7 days. **b**, The levels of HBV DNA and the cell viability were determined by RT-qPCR

1 and MTS assays in HepAD38 (Tet off) cells treated with D-TTK001 for 7 days. **c-f**, The levels of
2 HBV DNA, HBsAg, and HBeAg were evaluated by qPCR and ELISA assays in the supernatant of
3 HepAD38 (Tet off), dHepaRG and PHH cells. The cell viability was measured by MTS assay.
4 HepAD38 (Tet off) cells were infected with HBV and were continuously treated with D-TTK001,
5 ETV or IFN- α for 7 days. Data are represented as means $\pm$ SD (n=3). * p <0.05; ** p <0.01;
6 *** p <0.001 (Student's t test).

Fig. S3. Efficacy of D-TTK001 against HBV *in vivo*. **a**, Immunohistochemistry staining for hALB, HBsAg and HBcAg were measured in the liver tissues from HBV-Hu hep-URG mice. Scale bars: 50 μ m. **b**, A comparative evaluation of changes in weight of HBV-Hu hep-URG mice treated with drugs. **c**, The levels of HBsAg were tested by ECLIA assays in the serum of HBV-Hu hep-URG mice before and after drug treatment. **d**, The levels of HBeAg were determined by ECLIA assays in the serum of HBV-Hu hep-URG mice before and after treatment. **e-g**, The levels

1 of HBV mRNA fragments, such as pgRNA, S, C-E were measured in the liver tissues from HBV-
2 infected human liver-chimeric mice by RT-qPCR assays. GAPDH gene was served as the internal
3 reference. **h**, A model of hydrodynamic injection (HDI)-based HBV-carrier mice. **i**, A
4 comparative evaluation of changes in weight of HDI-based HBV carrier mice treated with drug. **j**,
5 HBV DNA (IU/mL) levels were tested by qPCR in the serum of HDI-based HBV carrier mice.
6 The HDI-based HBV carrier mice were given dose-administration once a day for 4 weeks. Serum
7 samples were collected before exposure (Day 0) and 1, 2, 3, 4 weeks post-exposure (Day7, Day14,
8 Day21, Day28), respectively. Data are represented as means $\pm$ SD (n=3). * p <0.05; ** p <0.01;
9 *** p <0.001 (Student's t test).

Fig. S4. The toxicity evaluation of D-TTK001 *in vivo*. **a**, A model showed the protocol of acute toxicity experiment. **b**, A comparative evaluation of changes in the weight of mice after the drug treatment. **c**, Pathological characteristics of the organs of mice with acute toxicity treatment were observed by H&E staining. **d**, A model showed the protocol of long-term toxicity experiment. **e**, A comparative evaluation of changes in the weight of rats after drug treatment. **f**, Pathological characteristics of the organs of rats with long-term toxicity treatment were observed by H&E

1 staining. Data are represented as means $\pm$ SD (n=3). * p <0.05; ** p <0.01; *** p <0.001 (Student's t
2 test).
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Fig. S5. D-TTK001 efficiently controls the levels of HBV cccDNA *in vitro* and *in vivo*. a-d, HBV cccDNA levels were measured by qPCR assays in the nucleus of HepAD38 (Tet off) and HBV *de novo* PHH#2, dHepaRG, and HepG2-NTCP cells treated with D-TTK001, ETV or IFN-α for 7 days. Data are represented as means ± SD (n=3). *p<0.05; **p<0.01; ***p<0.001 (Student's *t* test).

Fig. S6. The mechanisms of D-TTK001 against HBV. **a**, The effect of D-TTK001 on the expression of HBx was measured by Western blot analysis in HBV *de novo* HepG2-NTCP cells treated with D-TTK001 for 7 days. **b**, The interaction of p300 with exogenous HBx was examined by Co-IP

1 assays in HEK293T cells transfected with Flag-tagged HBx and treated with D-TTK001 for 7
2 days. **c**, The interaction of p300 with endogenous HBx was examined by Co-IP assays in HBV *de*
3 *nov* HepG2-NTCP cells treated with D-TTK001 for 7 days. **d**, The interaction of DDB1 with
4 exogenous HBx was tested by Co-IP assay in HepG2 cells transfected with Flag-tagged HBx and
5 treated with D-TTK001 for 7 days. **e**, The interaction of DDB1 with exogenous HBx was tested by
6 Co-IP assay in HBV *de novo* HepG2-NTCP cells treated with D-TTK001 for 7 days. **f**, A model
7 of D-TTK001 against HBV. D-TTK001 depresses cccDNA transcription through regulating the
8 epigenetic modification of cccDNA minichromosome and disturbing the interaction of HBx with
9 DDB1; and decreases the levels of cccDNA by down-regulating MSL2 which leads to the increase
10 of APOBEC3B in liver. **g-h**, The levels of HBV DNA, HBsAg and HBeAg were evaluated by
11 qPCR and ELISA assays in the supernatant of HepAD38 (tet off) and HBV *de novo* HepG2-
12 NTCP cells. The cells were continuously treated with D-TTK001 (200 μ M), D-TTK001-mut (200
13 μ M), ETV (100 nM) for 7 days. The pcDNA3.1-HBx and pcDNA3.1-Vector were transfected at
14 time points of day 0, day 2, and day 4. Data are represented as means $\pm$ SD (n=3). * p <0.05;
15 ** p <0.01; *** p <0.001 (Student's *t* test).

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Fig. S7. Efficacy of the combination of D-TTK001 with ETV or IFN-α *in vitro*. **a-c**, The levels of HBV DNA, HBsAg, and HBeAg were measured by qPCR and ELISA assays in the supernatant of HepAD38 (Tet off) cells treated with both D-TTK001 and ETV for 7 days. **d-f**, The levels of HBV DNA, HBsAg, and HBeAg were evaluated by qPCR and ELISA assays in the supernatant of HepAD38 (Tet off) cells treated with both D-TTK001 and IFN-α for 7 days. Data are represented as means ± SD (n=3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (Student's t test).

Fig. S8. A model of D-TTK001 against HBV. TTK001 is identified from the fragment of

HBXIP sequence by PPI strategy. All of the amino acids of TTK001 are reversed into the D-type

amino acids, termed D-TTK001. Mechanically, D-TTK001 depresses cccDNA transcription

1 through regulating the epigenetic modification of cccDNA minichromosome and disturbing the
2 interaction of HBx with DDB1; and decreases the levels of cccDNA by down-regulating MSL2
3 which leads to the increase of APOBEC3B in liver, contributing to the inhibition of HBV.

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1 **Table S1.** Pharmacokinetics of D-TTK001 in rats with subcutaneous injection

Parameters		Single subcutaneous administration					
Name	Units	1 mg/kg		2 mg/kg		4 mg/kg	
$C_{\max}$	ng/mL	857	± 178	1790	± 298	3610	± 734
$T_{\max}$	h	0.333	± 0.204	0.417	± 0.204	0.417	± 0.129
AUC _{0-6h}	h·ng/mL	1250	± 63.9	2570	± 194	5360	± 651
AUC _{0-∞}	h·ng/mL	1250	± 63.7	2570	± 193	5370	± 648
MRT _{0-6h}	h	1.14	± 0.154	1.06	± 0.146	1.11	± 0.176
MRT _{0-∞}	h	1.15	± 0.152	1.07	± 0.145	1.12	± 0.182
V_d	L/kg	0.555	± 0.116	0.45	± 0.0714	0.565	± 0.162
CL	L/h/kg	0.801	± 0.0398	0.781	± 0.0631	0.755	± 0.102
$t_{1/2}$	h	0.481	± 0.102	0.398	± 0.0474	0.515	± 0.113

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1 **Table S2.** Pharmacokinetic parameters for D-TTK001 after single and multiple doses in rats

Parameters		2 mg/kg			
Name	Units	Single injection Day1		Continuous injection Day7	
$C_{\max}$	ng/mL	1790	± 298	2360	± 123
$T_{\max}$	h	0.417	± 0.204	0.375	± 0.137
AUC_{0-6h}	h·ng/mL	2570	± 194	3060	± 169
$AUC_{0-\infty}$	h·ng/mL	2570	± 193	3060	± 170
$MRT_{0-\infty}$	h	1.07	± 0.145	0.955	± 0.0663
V_d	L/kg	0.45	± 0.0714	0.655	± 0.0373
CL	L/h/kg	0.781	± 0.0631	0.449	± 0.06
$t_{1/2}$	h	0.398	± 0.0474	0.476	± 0.0604

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1 **Table S3.** Pharmacokinetics of D-TTK001 in monkey with subcutaneous injection

Parameters		Single subcutaneous administration					
Name	Units	0.5 mg/kg		1 mg/kg		2 mg/kg	
$C_{\max}$	ng/mL	1260	± 250	2280	± 194	5400	± 881
$T_{\max}$	h	0.833	± 0.258	0.75	± 0.274	0.667	± 0.258
AUC _{0-6h}	h·ng/mL	3620	± 893	6380	± 442	14100	± 2490
AUC _{0-∞}	h·ng/mL	3620	± 895	6390	± 442	14100	± 2490
MRT _{0-6h}	h	1.97	± 0.292	1.99	± 0.2	1.88	± 0.147
MRT _{0-∞}	h	1.99	± 0.295	2	± 0.201	1.89	± 0.148
V _d	L/kg	0.245	± 0.0464	0.286	± 0.0496	0.254	± 0.0676
CL	L/h/kg	0.145	± 0.0366	0.157	± 0.0109	0.145	± 0.0245
$t_{1/2}$	h	1.19	± 0.196	1.27	± 0.233	1.2	± 0.216

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1 **Table S4.** Pharmacokinetic parameters for D-TTK001 after single and multiple doses in monkeys

Parameters		1 mg/kg			
Name	Units	Single injection Day1		Continuous injection Day7	
$C_{\max}$	ng/mL	2280	± 194	2470	± 236
$T_{\max}$	h	0.75	± 0.274	0.625	± 0.306
AUC_{0-6h}	h·ng/mL	6380	± 442	5870	± 462
$AUC_{0-\infty}$	h·ng/mL	6390	± 442	5880	± 462
$MRT_{0-\infty}$	h	2	± 0.201	1.78	± 0.176
V_d	L/kg	0.286	± 0.0496	0.353	± 0.117
CL	L/h/kg	0.157	± 0.0109	0.171	± 0.0141
$t_{1/2}$	h	1.27	± 0.233	1.43	± 0.439

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1 **Table S5.** Information of HBV-infected human liver-chimeric mice

Groups	Number	HSA (ng/ml)	HBV DNA (copy/ml) after HBV particles injection		
			4 week	5 week	6 week
D-TTK001 10 mg/kg	1	2,654,191.50	2.00E+06	2.42E+06	1.58E+06
	2	816,344.45	4.30E+05	7.11E+05	9.11E+05
	3	2,047,426.25	8.29E+03	5.77E+05	5.69E+05
	4	1,118,151.25	3.07E+05	4.38E+05	2.79E+05
	5	1,135,842.05	2.34E+05	2.57E+05	1.40E+05
D-TTK001 5 mg/kg	6	4,350,590.85	8.57E+06	2.18E+07	1.32E+07
	7	2,341,671.35	1.25E+05	5.71E+05	5.81E+05
	8	1,488,376.40	1.42E+05	6.22E+05	4.33E+05
	9	1,445,497.30	2.87E+05	2.93E+05	2.56E+05
	10	1,286,685.25	4.86E+05	1.63E+05	1.44E+05
D-TTK001 2.5 mg/kg	11	1,850,263.55	1.37E+06	3.91E+06	1.83E+06
	12	1,232,876.30	9.02E+05	8.51E+05	7.37E+05
	13	1,981,115.80	2.95E+05	4.80E+05	5.11E+05
	14	1,879,166.35	2.26E+05	1.37E+05	1.55E+05
	15	1,102,910.35	4.24E+05	3.89E+05	1.34E+05
ETV	16	4,330,688.85	2.92E+06	2.25E+06	1.86E+06
	17	3,180,305.40	6.27E+06	2.02E+06	9.24E+05
	18	1,927,548.95	6.89E+05	6.10E+05	4.34E+05
	19	1,245,156.35	4.76E+04	1.93E+05	1.89E+05
	20	1,291,550.10	8.23E+04	1.11E+05	1.32E+05
5% Glu	21	1,128,271.85	1.22E+06	6.38E+06	4.49E+06
	22	3,288,609.55	1.88E+06	1.06E+06	9.26E+05
	23	3,662,839.75	2.49E+05	6.94E+05	3.43E+05
	24	1,028,102.75	5.07E+05	9.41E+05	3.19E+05
	25	1,163,458.80	6.55E+04	1.37E+05	1.39E+05

2 Note: HAS is short for Human Serum Albumin.

3

1 **Table S6.** The influence of D-TTK001 on the hemogram index of mice

Index	Group No.	5% Glu			Mean	D-TK			Mean	P value
		1	2	3		1	2	3		
WBC ($\times 10^9/L$)	♂	8.08	8.63	8.06	8.26	8.63	8.68	8.8	8.70	0.0821
	♀	8.89	8.26	8.16	8.44	8.11	8.41	8.13	8.22	0.4254
RBC ($\times 10^9/L$)	♂	7.76	7.27	7.62	7.55	7.63	7.65	7.49	7.59	0.8081
	♀	7.36	7.56	7.5	7.47	7.96	7.29	7.69	7.65	0.4422
HGB ($\times 10^2 g/L$)	♂	1.37	1.3	1.4	1.36	1.33	1.4	1.35	1.36	0.9311
	♀	1.4	1.4	1.43	1.41	1.33	1.4	1.38	1.37	0.1583
HCT (%)	♂	26.48	28.86	27.66	27.67	26.1	27.44	26.4	26.65	0.2703
	♀	28.98	27.31	26.42	27.57	28.58	26.69	28.55	27.94	0.7240
PLT ($\times 10^8/L$)	♂	6.22	6.26	6.1	6.19	6.21	6.2	6.3	6.24	0.4940
	♀	6.21	6.12	6.24	6.19	6.23	6.1	6.27	6.20	0.8810
PCT (%)	♂	0.29	0.29	0.27	0.28	0.3	0.29	0.27	0.29	0.7780
	♀	0.3	0.27	0.28	0.28	0.28	0.28	0.3	0.29	0.7780
PDW (%)	♂	17.97	17.37	17.97	17.77	17.07	17.57	17.48	17.37	0.1911
	♀	17.83	17.79	17.56	17.73	17.13	17	17.97	17.37	0.3174
MPV (fL)	♂	4.55	4.59	4.56	4.57	4.51	4.56	4.51	4.53	0.1234
	♀	4.52	4.63	4.62	4.59	4.54	4.55	4.65	4.58	0.8503
MCV (fL)	♂	37.88	37.58	37.73	37.73	36.2	37.6	36.9	36.90	0.1150
	♀	36.97	36.02	36.89	36.63	36.6	36.97	36.47	36.68	0.8826

2 Abbreviation: WBC, White Blood Cell Count; RBC, Red Blood Count; HGB, Hemoglobin; HCT,

3 Hematocrit; PLT, Platelet; PCT, Thrombolytic; PDW, Platelet Distribution Width; MPV, Mean

4 Platelet Volume; MCV, Mean Corpuscular Volume.

5

1 **Table S7.** The influence of D-TTK001 on the hematology and coagulation of rats

Index	Group No.	5% Glu			Mean	D-TK				Mean	P value
		1	2	3		1	2	3	4		
WBC ($\times 10^9/L$)	♂	31.44	17.36	3.51	6.55	26.91	19.96	16.3	15.07	19.56	0.7869
	♀	26.71	39.27	20.92	28.97	14.74	18.76	15.78	19.37	17.16	0.0545
RBC ($\times 10^{12}/L$)	♂	7.46	8.33	7.06	7.62	8.16	7.9	7.62	8.76	8.11	0.2987
	♀	7.63	7.49	7.78	7.63	7.91	8.23	8.53	8.29	8.24 ^a	0.0149
HGB (g/L)	♂	147	161	141	150	154	159	159	171	161	0.1513
	♀	150	142	147	146	145	150	158	150	151	0.2899
HCT (%)	♂	45.1	49.7	43.8	46.2	48.7	47.3	48.3	52	49.1	0.195
	♀	45	43.8	44.5	44.4	44.5	46.8	47.7	46.3	46.3	0.0763
MCV (fL)	♂	60.5	59.7	62.1	60.8	59.7	59.8	63.4	59.3	60.6	0.8718
	♀	58.9	58.5	57.3	58.2	56.2	56.9	55.9	55.9	56.2 ^b	0.0093
MCH (pg)	♂	19.7	19.4	20	19.7	18.9	20.1	20.8	19.5	19.8	0.8136
	♀	19.6	19	18.9	19.2	18.4	18.2	18.5	18.1	18.3 ^b	0.0095
MCHC (g/L)	♂	326	325	322	324	316	336	329	330	328	0.5312
	♀	333	325	330	329	327	320	330	324	325	0.2572
PLT ($\times 10^9/L$)	♂	1473	1037	1626	1379	1135	996	943	1116	1047	0.0895
	♀	1552	1359	1875	1595	968	695	1160	1121	986 ^a	0.0185
N (%)	♂	26.7	8.2	8.5	14.5	7.2	5.6	10.6	9.8	8.3	0.2969
	♀	11.5	9.9	3.4	8.3	7.3	6	8.6	8.4	7.6	0.7648
L (%)	♂	69	84.7	87.2	80.3	87.8	87.8	74.4	83.8	83.5	0.6253
	♀	83.6	85.7	92.9	87.4	78	88	85	85.1	84	0.3724
M (%)	♂	2.5	2.8	1.3	2.2	1.8	2	3	2.9	2.4	0.6876
	♀	2.4	1.5	1.4	1.8	2.9	2.9	2.4	2.7	2.7 ^a	0.0243
E (%)	♂	0.3	0.3	1.5	0.7	0.2	0.3	0.5	0.2	0.3	0.2986
	♀	0.6	0.2	0.7	0.5	0.5	0.7	0.7	0.9	0.7	0.2672
Ret (%)	♂	6.06	3.82	8.16	6.01	5.27	4.84	4.13	3.23	4.37	0.2198
	♀	7.9	7.29	5.3	6.83	4.77	4.33	5	6.18	5.07	0.0808
PT (s)	♂	18.3	32	14.3	21.5	19.6	21.5	18.8	16.7	19.2	0.6291
	♀	17.3	14.1	14.7	15.4	13.7	15.4	15.6	14.9	14.9	0.6488
APTT (s)	♂	25.2	30.8	21.9	26	25.1	25.9	25.6	26.2	25.7	0.9079
	♀	27	23.9	23.7	24.9	22.1	21.3	22.7	22.8	22.2 ^a	0.0434

2 NOTE: a, $p < 0.05$; b, $p < 0.01$, vs 5% Glu group.

3 Abbreviation: WBC, White Blood Cell Count; RBC, Red Blood Count; HGB, Hemoglobin; HCT,
4 Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC,
5 Mean Corpuscular Hemoglobin Concentration; PLT, Platelet; N, Neutrophil; L, Lymphocyte; M,
6 Monocytes; E, Eosinophil; Ret, Reticulocyte; PT, Prothrombin Time; APTT, The Activated
7 Partial Thromboplastin Time.

8

1 **Table S8.** The influence of D-TTK001 on serum biochemical index of rats

Index	Group	5% Glu			Mean	D-TK				Mean	P value
		1	2	3		1	2	3	4		
ALT	♂	31.1	38.5	30.6	33.4	39.1	46.2	57.4	58.5	50.3*	0.0354
(U/L)	♀	29.1	27.2	27.3	27.9	31.3	31.8	25.4	26	28.6	0.7294
AST	♂	172.4	229.4	173.7	191.8	193.4	199.2	207.2	207.4	201.8	0.5658
(U/L)	♀	177.9	149.7	223.6	183.7	187.3	125.9	174.7	192.9	170.2	0.6183
ALP	♂	190.9	187.7	164.5	181	267.8	319.5	169.9	243.9	250.3	0.1236
(U/L)	♀	67.7	100.4	86.2	84.8	66.8	81.3	91.2	101.3	85.2	0.9753
BUN	♂	5.83	8.56	7.35	7.25	7.81	7.22	6.9	8.24	7.54	0.7094
(mmol/L)	♀	6.66	6.87	6.13	6.55	8.32	11.47	8.16	9.06	9.25*	0.0329
Crea	♂	50.4	61.4	67.1	59.6	66.7	56.5	58.4	59.1	60.2	0.916
(μmol/L)	♀	61.8	75.4	64.4	67.2	58.4	72.4	62.9	64	64.4	0.5964
TP	♂	55.4	52	58.3	55.2	58.1	56.1	52.7	56.2	55.8	0.7995
(g/L)	♀	60.8	55.8	61.5	59.4	58.7	51.6	58.3	58.2	56.7	0.3372
ALB	♂	19.2	18.5	20.5	19.4	21.5	22.7	19.1	20.1	20.9	0.2275
(g/L)	♀	22.3	20.8	24.4	22.5	22.6	18.9	22	24	21.9	0.7025
GLU	♂	6.84	6.92	7.24	7	7.6	6.11	8.15	7.18	7.26	0.6389
(mmol/L)	♀	7.05	11.19	8.67	8.97	6.95	6.9	6.68	8.2	7.18	0.161
TCHO	♂	1.38	0.75	1.84	1.32	1.57	1.74	1.16	1.82	1.57	0.4667
(mmol/L)	♀	1.69	1.2	1.84	1.58	1.66	1.37	1.15	1.58	1.44	0.5455
TBIL	♂	0.33	0.69	0.6	0.54	0.49	0.77	0.62	0.64	0.63	0.4622
(μmol/L)	♀	0.47	0.44	0.14	0.35	0.5	0.46	0.57	0.65	0.55	0.1115
Tg	♂	0.33	0.26	0.48	0.36	0.4	0.43	0.54	0.71	0.52	0.1604
(mmol/L)	♀	0.3	0.37	0.24	0.3	0.24	0.34	0.33	0.36	0.32	0.7629
CK	♂	1215.8	1573.7	1316	1368.5	1406	1437.7	1328.3	2507.1	1669.8	0.4201
(U/L)	♀	1140.9	765	1668.8	1191.6	1189.8	568.8	1062.7	1127.8	987.3	0.4927
γ-GT	♂	1.1	1.8	1.9	1.6	1.9	1.5	2.2	1.2	1.7	0.7771
(U/L)	♀	1.1	1.4	1.3	1.3	1.9	1.9	1.7	1.4	1.7*	0.0338
LDH	♂	2165.3	2745.8	1969.4	2293.5	2110.4	2043.3	2061.7	1953.3	2042.2	0.262
(U/L)	♀	1968.9	1585.7	2636.9	2063.8	2054.9	1084.8	2097.2	2301.4	1884.6	0.6815
GLOB	♂	36.2	33.5	37.8	35.8	36.6	33.4	33.6	36.1	34.9	0.5552
(g/L)	♀	38.5.0	35	37.1	36.9	36.1	32.7	36.3	34.2	34.8	0.1823
A/G	♂	0.53	0.55	0.54	0.54	0.59	0.68	0.57	0.56	0.6	0.1265
	♀	0.58	0.59	0.66	0.61	0.63	0.58	0.61	0.7	0.63	0.61
AST	♂	5.54	5.96	5.68	5.73	4.95	4.31	3.61	3.55	4.11*	0.0102
/ALT	♀	6.11	5.5	8.19	6.6	5.98	3.96	6.88	7.42	6.06	0.6525

2 NOTE: a, p<0.05, vs 5% Glu group.

3 Abbreviation: ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; ALP, Alkaline
4 Phosphatase; BUN, Blood Urea Nitrogen; Crea, Creatinine; TP, Total Protein; ALB, Albumin;
5 GLU, Glucose; TCHO, Total Cholesterol; TBIL, Total Bilirubin; Tg, Triglyceride; CK, Creatine
6 Kinase; γ-GT, Gamma-Glutamyltransferase; LDH, Lactate Dehydrogenase; GLOB, Globulin;
7 A/G, Albumin/ Globulin.

8

1 **Table S9.** The influence of D-TTK001 on blood electrolyte of rats

Group	No./ Gender	K ⁺ (mmol/L)	Na ⁺ (mmol/L)	Cl ⁻ (mmol/L)	No./ Gender	K ⁺ (mmol/L)	Na ⁺ (mmol/L)	Cl ⁻ (mmol/L)
5%Glu	1♂	4.53	139.4	105	1♀	4.37	139.4	106.7
	2♂	4.4	139.6	106.1	2♀	4.15	137.2	105
	3♂	4.29	140.7	106	3♀	4.54	140.2	107.5
Mean		4.41	139.9	105.7			4.35	138.9
SD		0.12	0.7	0.6			0.2	1.6
D-TK	1♂	4.22	141.2	107.2	1♀	4.33	139.6	108.1
	2♂	4.56	139.9	106.1	2♀	4.09	140.4	109.6
	3♂	4.03	138.3	105.3	3♀	4.43	139.9	109.1
	4♂	4.62	139.4	107.8	4♀	4.18	138.9	107.4
Mean		4.36	139.7	106.6			4.26	139.7
SD		0.28	1.2	1.1			0.15	0.6
P value		0.7909	0.8097	0.2683			0.4951	0.4018
								0.0525

2 Abbreviation: K⁺, Potassium ion; Na⁺, Sodium ion, Cl⁻, Chloride ion.

3

1 **Table S10.** The influence of D-TTK001 on organ weight of rats (g)

Index	Group No.	5% Glu			Mean	D-TK				Mean	P value
		1	2	3		1	2	3	4		
Heart	♂	1.273	1.059	1.313	1.215	1.119	1.269	1.152	1.109	1.162	0.534
	♀	0.848	0.917	0.909	0.891	0.984	0.878	0.953	1.06	0.969	0.169
Liver	♂	10.813	8.923	11.548	10.428	8.995	10.187	10.504	8.993	9.67	0.389
	♀	7.615	8.102	6.723	7.48	7.35	5.784	7.315	7.529	6.995	0.446
Spleen	♂	1.199	1.046	1.135	1.127	0.916	1.098	1.251	0.813	1.02	0.415
	♀	0.971	0.815	0.8	0.862	0.754	0.527	0.817	0.758	0.714	0.155
Kidneys	♂	2.557	2.082	2.369	2.336	2.402	2.647	2.537	2.206	2.448	0.518
	♀	1.75	1.752	1.929	1.81	1.753	1.432	1.785	1.866	1.709	0.448
Thymus	♂	0.7	0.386	2.894	1.327	0.687	0.515	0.426	0.391	0.505	0.407
	♀	0.471	0.458	0.494	0.474	0.398	0.317	0.565	0.421	0.425	0.463
Testicles	♂	3.456	3.139	3.093	3.229	2.905	2.953	3.231	3.146	3.059	0.254
	♀	0.69	0.486	0.565	0.58	0.466	0.376	0.374	0.65	0.467	0.268
Epididymis	♂	1.002	0.897	0.761	0.887	0.775	0.811	0.914	1.007	0.877	0.912
	♀	0.208	0.166	0.174	0.183	0.163	0.124	0.159	0.137	0.146	0.061

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3

1 **Table S11.** The influence of D-TTK001 on organ coefficients of rats (organ weight/body weight,
2 mg/g)

Index	Group No.	5% Glu			Mean	D-TK				Mean	P value
		1	2	3		1	2	3	4		
Heart	♂	3.669	3.461	3.955	3.695	3.508	3.711	3.692	3.837	3.687	0.959
	♀	3.49	3.97	3.918	3.793	4.297	4.646	4.143	4.732	4.455 ^a	0.025
Liver	♂	31.2	29.2	34.8	31.7	28.2	29.8	33.7	31.1	30.7	0.625
	♀	31.3	35.1	29	31.8	32.1	30.6	31.8	33.6	32	0.894
Spleen	♂	3.455	3.418	3.419	3.431	2.871	3.211	4.01	2.813	3.226	0.512
	♀	3.996	3.528	3.448	3.657	3.293	2.788	3.552	3.384	3.254	0.156
Kidneys	♂	7.369	6.804	7.136	7.103	7.53	7.74	8.131	7.633	7.759 ^a	0.025
	♀	7.202	7.584	8.315	7.7	7.655	7.577	7.761	8.33	7.831	0.717
Thymus	♂	2.017	1.261	8.717	3.999	2.154	1.506	1.365	1.353	1.594	0.417
	♀	1.938	1.983	2.129	2.017	1.738	1.677	2.457	1.879	1.938	0.73
Testicles	♂	9.96	10.258	9.316	9.845	9.107	8.635	10.356	10.886	9.746	0.887
	♀	2.84	2.104	2.435	2.46	2.035	1.989	1.626	2.902	2.138	0.42
Epididymis	♂	2.888	2.931	2.292	2.704	2.429	2.371	2.929	3.484	2.804	0.7877
	♀	0.856	0.719	0.75	0.775	0.712	0.656	0.691	0.612	0.668	0.0566

1 **Table S12.** List of primers used in this paper

Primer		Sequence (5'-3')
mouse-Gapdh	Forward	CCTGCCAAGTATGATGACAT
	Reverse	GTTGCTGTAGCCGTATTCA
GAPDH	Forward	GAGTCAACGGATTTGGTCGT
	Reverse	TTGATTTTGGAGGGATCTCG
HBV X (HBx/pgRNA)	Forward	ATGGCTGCTAGGCTGTGCTG
	Reverse	TTAGGCAGAGGTGAAAAAGT
pgRNA	Forward	CTCCTCCAGCTTATAGACC
	Reverse	GTGAGTGGGCCTACAAA
HBV S (HBs mRNA)	Forward	CCTGTATTCCCATCCCATC
	Reverse	TAGGGTTTAAATGTATACCC
HBV e-c (HBc/HBe mRNA)	Forward	TCAATCTCGGGAATCTCAATGTTA
	Reverse	CCTAGCAGGCATAATCAATTGCAG
HBV	Forward	CCTAGTAGTCAGTTATGTCAAC
	Reverse	TCTATAAGCTGGAGGAGTGCGA
cccDNA	Forward	CGTCTGTGCCTTCTCATCTGC
	Reverse	GCACAGCTTGGAGGCTTGAA
chip-cccDNA	Forward	CTCCCCGTCTGTGCCTTCT
	Reverse	GCCCCAAAGCCACCCAAG

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3

1 **Table S13.** List of antibodies used in this paper

Antibody	Manufacture	Catalog Number
HBx Polyclonal Antibody	Abcam	ab39716
Ki67 Polyclonal Antibody	Proteintech	19972-1-AP
Anti-GST-tag pAb	MBL	PM013
Anti-His-tag mAb	MBL	D291-3
Anti-HA-tag mAb	MBL	M180-3
Anti-DDDDK-tag-mAb	MBL	M185-3L
Anti-HBXIP antibody	Abcam	ab157480
Anti-Histone H3 antibody	Abcam	ab1791
LaminB1	proteintech	12987-1-AP
Anti-DDB1 antibody	Abcam	ab109027
Anti-KAT3B/p300 antibody	Abcam	ab14984
CBP	Santa Cruz	sc-365387
Anti-HDAC1 antibody	Abcam	ab1767
Anti-SIRT1 antibody	Abcam	ab110304
Anti-EZH2 antibody	Abcam	ab191250
SMC6 Polyclonal Antibody	Abcam	ab155495
APOBEC3B antibody	Abcam	ab184990
MSL2 antibody	Novus	H00055167- D01P
SMC5 Polyclonal Antibody	Abcam	ab18038
HBsAg Monoclonal Antibody	Abcam	ab68518
HBcAg Monoclonal Antibody	Abcam	ab8639
β -Actin Monoclonal Antibody	Sigma-Aldrich	A2228

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3

Figures

Figure 1

A new D-type polypeptide derived from HBXIP sequence is engineered. a, A model of strategy against HBV for TTK001. b, Affinity analysis of HBx binding to TTK001 using SPR. c, Affinity analysis of HBx binding to D-TTK001 using SPR. d, The interaction of HBx with D-TTK001 was tested by GST-Pull-down assays. e,

Co-IP assays to test the binding of HBx and D-TTK001. HEK293T cells were transfected with plasmids Flag-HBx, and cells were incubated with HA-tagged TTK001 polypeptide. The interaction of HBx with TTK001 was examined by Co-IP and immunoblotting. f, The effect of D-TTK001 on the binding of HBXIP with exogenous HBx was measured by Co-IP assays in HEK293T cells transfected with plasmids Flag-HBx and treated with D-TTK001 (200 μ M) for 7 days. g, The effect of D-TTK001 on the binding of HBXIP with endogenous HBx was measured by Co-IP assays in HBV de novo HepG2-NTCP cells treated with D-TTK001 (200 μ M) for 7 days. h-i, Immunofluorescence analysis of D-TTK001 (red) in HepG2.2.15 and HepAD38 (Tet off) cells. HepG2.2.15 (h) and HepAD38 (i) cells were treated with red luciferin cy3-labeled D-TTK001 (100 μ M) and determined by immunofluorescence assays at different time points. DAPI (blue) stained the nuclei. Scale bars, 10 μ m.

Figure 2

Efficacy of D-TTK001 against HBV in vitro. a, The levels of HBV DNA were measured by qPCR in the supernatant of HBV de novo PHH cells. b, PHH cell viability was measured by MTS assay. c, The levels of HBsAg and HBeAg were examined by ELISA in the supernatant of HBV de novo PHH cells. d, The levels of HBcAg were determined by immunofluorescence staining in HBV de novo PHH cells. Scale bars, 50 μ m. e, The levels of HBV DNA, HBsAg, and HBeAg were tested by qPCR and ELISA assays in the supernatant of HBV de novo PHH cells. f, The levels of HBV DNA were measured by qPCR in the supernatant of HBV de novo HepG2-NTCP cells. g, HepG2-NTCP cell viability was evaluated by MTS assays. h, The levels of HBsAg and HBeAg were examined by ELISA in the supernatant of HBV de novo HepG2-NTCP cells. i, The level of HBcAg was measured by immunofluorescence staining in HBV de novo HepG2-NTCP cells. Scale bars, 50 μ m. j, The levels of HBV DNA, HBsAg, and HBeAg were determined by qPCR and ELISA assays in the supernatant of HBV de novo HepG2-NTCP cells. Data are represented as means $\pm$ SD (n=3). ns, no significant; *p<0.05; **p<0.01; ***p<0.001 (Student's t test).

Figure 3

Efficacy of D-TTK001 against HBV in HBV-infected human liver-chimeric mice. a, A model of HBV-infected human liver-chimeric mice (HBV-HuHep-URG mice). b-c, The level of HBV DNA was tested by qPCR in the serum of human liver-chimeric mice. The HBV-HuHep-URG mice were given dose-administration (2.5, 5, 10 mg/kg) once a day for 4 weeks. Serum samples were collected before exposure (Day 0) and 1, 2, 3, 4 weeks post-exposure (Day 7, Day 14, Day 21, Day 28), respectively. d, Pathological observation and immunohistochemistry staining for HBsAg and HBcAg were measured in the liver tissues from HBV-

Huhep-URG mice with different treatments. Scale bars: 20 μm . e-f, The levels of ALT and AST were evaluated by ALT/AST detection kits in the serum of HBV-Huhep-URG mice. The HBV-Huhep-URG mice were given dose-administration once a day for 4 weeks. Serum samples were collected after exposure (Day 28). Data are represented as means $\pm$ SD (n=3). *p<0.05; **p<0.01; ***p<0.001 (Student's t test).

Figure 4

D-TTK001 efficiently controls the levels of HBV cccDNA in vitro and in vivo. a, The cccDNA levels were measured by qPCR assays in the nucleus of HBV *de novo* PHH cells treated with D-TTK001, ETV or IFN- α for 7 days. b, The cccDNA levels were examined by Southern blot analysis in the nucleus of HBV *de novo*

PHH cells treated with D-TTK001 (200 μ M), ETV (100 nM), and IFN- α (2000 IU/mL) for 7 days. c, The cccDNA levels were measured by Southern blot analysis in the nucleus of cells of HepAD38 (Tet off), HBV de novo PHH, dHepaRG, and HepG2-NTCP treated with D-TTK001 (200 μ M) for 7 days. d, The cccDNA levels were determined by qPCR assays in the liver of HBV-HuHep-URG mice treated with D-TTK001 (10, 5, 2.5 mg/kg) and ETV (0.076 mg/kg) for 28 days. e, The cccDNA levels were tested by Southern blot analysis in the liver of HBV-HuHep-URG mice. Data are represented as means $\pm$ SD. * p <0.05; ** p <0.01; *** p <0.001 (Student's t test).

Figure 5

The mechanisms of D-TTK001 against HBV. a, Immunofluorescence staining was used to identify the location of D-TTK001 (red) and HBx (green) in HepAD38 (Tet off) and HepG2 cells treated with or without Red luciferin cy3-labeled D-TTK001 (100 μ M). DAPI (blue) stained the nuclei. Scale bars: 10 μ m. b-c, The effect of D-TTK001 on the expression of HBx was tested by Western blot analysis in HepAD38 (Tet off) and HBV de novo PHH cells treated with D-TTK001 for 7 days. d-e, The assembly of histone H3, AcH3, H3K4me3, H3K9me3, H3K27me3, EP300, CBP, HDAC1, SIRT1, and EZH2 onto cccDNA was determined by ChIP-qPCR in HepAD38 (Tet off) or HBV de novo HepG2-NTCP cells treated with D-TTK001 for 7 days, respectively. f, The interaction of DDB1 with exogenous HBx was examined by Co-IP assay in HEK293T cells transfected with Flag-tagged HBx and treated with D-TTK001 for 7 days. g, The effect of D-TTK001 on the expression of SMC5, SMC6, MSL2 and APOBEC3B was evaluated by Western blot analysis in HBV de novo HepG2-NTCP cells treated with D-TTK001 for 7 days. h, The effect of D-TTK001 on the expression of SMC5, SMC6, MSL2 and APOBEC3B was evaluated by Western blot analysis in HepAD38 cells treated with D-TTK001 for 7 days. i, The levels of HBV DNA, HBsAg, and HBeAg were measured by qPCR and ELISA assays in the supernatant of HBV de novo PHH cells treated with D-TTK001 (200 μ M), D-TTK001-mut (200 μ M), ETV (100 nM) for 7 days. The pcDNA3.1-HBx (2 μ g) and pcDNA3.1-Vector (2 μ g) were transfected at time points of day 0, day 2, and day 4. Data are represented as means $\pm$ SD (n=3). *p<0.05; **p<0.01; ***p<0.001 (Student's t test).

Figure 6

Efficacy of the combination of D-TTK001 with ETV or IFN-α in vitro. a-c, The levels of HBV DNA, HBsAg, and HBeAg were measured by qPCR and ELISA assays in the supernatant of HepAD38 (Tet off) cells treated with both D-TTK001 and ETV for 7 days. d, HepAD38 cell viability was tested by MTS assays. e-g, The levels of HBV DNA, HBsAg, and HBeAg were evaluated by qPCR and ELISA assays in the supernatant of HepAD38 (Tet off) cells treated with both D-TTK001 and IFN-α for 7 days. (H) HepAD38 cell viability was tested by MTS assays. Data are represented as means ± SD (n=3). *p<0.05; **p<0.01; ***p<0.001 (Student's t test).