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Heterogeneous nuclear ribonucleoprotein A2B1, a novel drug target for hepatitis B virus infection

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Abstract

HBV infection is a major global health burden that needs novel immunotherapeutic approaches. Herein, we show that heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) is a novel drug target for HBV infection. We reveal the new target with highly selective probes of PAC5, a natural sesquiterpene derivative. PAC5 show potent anti-HBV activity in vivo and in vitro. Further studies on its mode of action indicate that PAC5 binds to the residue Asp49 and a deep groove in the RNA recognition motif1 (RRM1) region of hnRNPA2B1. PAC5-bound hnRNPA2B1 is activated, dimerized, and translocated to the cytoplasm where it activates the TBK1-IRF3 pathway, leading to the production of type I interferons (IFNs). Furthermore, PAC5 also suppresses other viral replications, such as SARS-CoV-2 and vesicular stomatitis virus (VSV). Our results indicate that PAC5 is the first small molecule agonist of hnRNPA2B1, a drug target potentially valid for broad-spectrum viral infections, providing a novel strategy for viral immunotherapy.
**Background**

Hepatitis B virus (HBV) infection is a major public health problem with high prevalence in the African, Eastern Mediterranean, South-East Asia, and Western Pacific regions. Current therapies for HBV infection, such as pegylated-interferon-α (Peg-IFNα) and nucleos(t)ide analogues (NAs), are not suitably efficient in providing functional cure. Peg-IFNα is able to achieve sustained off-treatment control, but its success is limited to a minority of patients and is associated with side effects and toxicity. NAs therapy has been shown to potently suppress reverse transcription of pregenomic RNA and synthesis of HBV DNA in the cytoplasm, but the viral episomal covalently closed circular DNA (cccDNA) cannot fully eliminate in the nucleus, resulting in viral relapse after the cessation of therapy frequently. Of note, the majority of immunocompetent adults can clear HBV infection spontaneously, indicating that host innate immune responses are crucial for controlling HBV infection. Numerous preclinical and clinical studies have demonstrated that the intrahepatic activation of innate immunity by agonists efficiently blocks HBV infection and restores antiviral immunity. Therefore, agonistic activation of intrahepatic innate immune response, most likely in combination with current standard treatment, ensures a great promise for the therapy of HBV infection.

Heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) functions as a downstream effector of the N⁶-methyladenosine (m⁶A) mark and plays critical roles in RNA biology and the regulation of transcriptional responses.
studies suggest that hnRNPA2B1 plays an important role in antiviral innate immune response. As an RNA-binding protein, hnRNPA2B1 affects the polyribosomal distribution of HIV-1 RNA by modulating nuclear and cytoplasmic trafficking events. Wang et al. recently identified hnRNPA2B1 as a new nuclear sensor of both self- and pathogen-derived DNA.

In this study, we synthesized a new anti-HBV chemical series based on structure-activity relationships (SARs) of a series anti-HBV norbisabolane sesquiterpene derivatives from Phyllanthus spp. The herbs were traditionally used to treat HBV infection by local people in China. The best compound (PAC5) from this series is efficacious in HBV replication mice models, as indicated by a significant reduction in the synthesis of HBV DNA and viral proteins. Biochemical studies determined that PAC5 can bind with hnRNPA2B1 in the hepatocyte nucleus. Furthermore, we found that PAC5 binds to residue Asp49 and a deep groove in the RRM1 region of hnRNPA2B1 to promote the formation of hnRNPA2B1 dimer in the nucleus and translocation to the cytoplasm, leading to the production of type I IFNs. Importantly, we also show PAC5 inhibits other viruses, such as SARS-CoV-2 and vesicular stomatitis virus (VSV). Our study indicates that PAC5 is a highly selective and potent agonist for pathogen nucleos(t)ide sensor hnRNPA2B1, which may serve as a new preclinical broad-spectrum drug target for antiviral immunotherapy.

**Result**

**PAC5 inhibits HBV infection**
Phyllanthacidoid A (PA) is a structurally unique and complex anti-HBV norbisabolane sesquiterpene (Figure S1a and Table S1) that was originally isolated and identified from a *Phyllanthus* spp. used to treat HBV infection by local people of Yunnan province, China. Our prior structure-activity relationship (SAR) study of PA derivatives, suggested that the ester substitution (C-15) is critical to the antiviral activity. Thus, we here hydrolyzed PA to obtain the core structure of the norbisabolane sesquiterpene series (Figure S1a-c). Conversion of the carboxylic acid (C-15) to an aliphatic amide resulted in derivatives inhibiting the secretion of HBsAg and HBeAg better than those of PA (Table S1). Among them, the N-pentylamide substituent resulted in the key compound PAC5 (compound 3d) (Figure 1a and Figure S1). PAC5 showed potent antiviral activity with an IC$_{50}$ of 810 nM (inhibition of HBsAg) and no cytotoxicity in the HepG2 cell lines (SI >1000) (Table S1 and Figure 1b). Compare with PA, PAC5 showed 100-fold increase in activity and 1,000-fold decrease in toxicity (Table S1). Next, we examined the effect of PAC5 on inhibition of HBV *in vivo* (Figure 1c-g). Mice were pre-injected with rAAV8-1.3HBV into the tail vein to make a mice model for *in vivo* infection with HBV. Then, HBV infected mice treated with PAC5 (at 20 and 100 mg/kg dose), lamivudine (3-TC, at 50 mg/kg dose), 3TC (at 50 mg/kg dose) combined PAC5 (at 50 mg/kg dose), and physiological saline once per day for 8 weeks, respectively. As shown in Figure 1c, at 8 weeks post-infection, PAC5 reduced HBV titers by 1.8–2.2 log$_{10}$ versus infected placebo-treated mice at 20 mg/kg.
dose. At 100 mg/kg dose, PAC5 reduced HBV log$_{10}$ titers by 2.9-fold versus placebo. Next, at 8 weeks, after withdrawing PAC5 and 3-TC, PAC5-treated mice exhibited continuously reduced HBV titers, while 3-TC-treated mice showed obvious rebound in HBV titers. The virus-infected mice receiving combination therapy with PAC5 and 3-TC also displayed continuously reduced HBV log$_{10}$ titers by 2.9-fold versus placebo. PAC5 also inhibited the secretion of HBsAg and HBeAg in mice plasma, while 3-TC showed no inhibition.

Histopathological analysis was performed for the livers of mice. The placebo- and 3-TC-treated mice showed obvious necrotic focuses of hepatocytes with marked inflammatory cell infiltration, whereas PAC5-treated animals exhibited few necrotic focuses of hepatocytes with limited inflammatory cell infiltration (Figure 1d). Histopathological analysis and western blot assay showed PAC5 extensively inhibited the hepatic expression of hepatitis B virus core antigen (HBcAg) (Figure S2a-b). Accordingly, the key liver function index significantly decreased in the PAC5-treated mice versus the placebo-treated controls (Figure S2c). Further, mice were injected with PBSK-rtM204I plasmid via tail vein to establish a mice model for in vivo infection with drug-resistant HBV. As shown, PAC5 reduced HBV titers by 3.5 log$_{10}$ versus infected placebo-treated mice at 50 mg/kg dose, while 3-TC showed no antiviral activity (Figure 1e). Similarly, PAC5-treated animals exhibited few necrotic focuses of hepatocytes with minimal inflammatory cell infiltration (Figure 1f). Accordingly, key liver function index decreased in the PAC5-treated mice versus the placebo-treated
controls (Figure 1g). Also, PAC5 strongly inhibited the expression of HBcAg in the liver (Figure S2d-e). Lastly, PAC5 was evaluated for its toxicity in KM mice. In an acute toxicity experiment, no mice died after orally (p.o.) (2000 mg/kg) treatment with PAC5. In a repeated dose toxicity study, administration with PAC5 by p.o. 2000 mg/kg/d twice daily for 14 consecutive days did not result in noticeable toxicity in the mice (Table S2).

PAC5 targets hnRNPA2B1

To explore the functional target(s) of anti-HBV PAC5, we prepared chemical probes for affinity purification. In this experiment, PAC3, an inactive analogue of PAC5, was taken as a negative control. We synthesized both a positive probe (Probe) and a negative probe (NC) from PAC5 and PAC3, respectively, with biotin and an ethylene glycol linker (Figure 2a and Figure S1d). The blank control (BC) was also synthesized, only with biotin and ethylene glycol. Biological evaluation of Probe, NC, and BC showed that the biotin-tagged positive probe (PAC5) retained anti-HBV activity (Figure S3a-b, and Table S1), while the biotin-tagged negative probe (NC) and BC completely lost the activity (IC$_{50} >$100 μM) (Figure S3c-d).

Then, we performed pull-down experiments using Probe, NC and BC, respectively. As shown in Figure 2b, one clear band observed at around 70 kDa was specifically precipitated by the positive probe but not by NC and BC. Peptide mass fingerprinting data analysis revealed that a total of 117 specific proteins were identified in the Probe-treated sample (Figure 2c, and Figure
Among them, RPL5, hnRNPA2B1, SERPINA1, DNAJB12, and TALDO1 were the top five proteins with the highest abundance (Figure 2d). We further used immunoprecipitation (IP) assays to monitor the target proteins. Among them, only hnRNPA2B1 was specifically pulled down by the probe (Figure 2e). To further confirm whether PAC5 directly binds to hnRNPA2B1, we synthesized the fluorescence probe with a rhodamine tag attached to PAC5 (Figure 2f, S1e and S5), as well as negative control (NC, PAC3 attached with a rhodamine tag). A fluorescence probe exhibited a significant colocalization with hnRNPA2B1 in the nucleus (Figure 2g). hnRNPA2B1 has two isoforms, namely A2 and B1, which are the products of the alternative splicing of the precursor mRNA of the same gene. Interestingly, the coimmunoprecipitation assay showed that PAC5 bound with hnRNPB1 but not hnRNPA2 (Figure 2h).

The binding mode of PAC5 was studied through pocket identification using molecular docking, and point mutation experiments. Two potential binding pockets of PAC5 (pocket 0 and pocket 4) were found in protein hnRNPA2B1 (see Method and Figure S6). Pocket 0 and pocket 4 are located in the region of RNA recognition motif2 (RRM2) and motif1 (RRM1), respectively. Based on the docking results, residues Pro105 and Asp49 are key residues for PAC5 binding to pocket 0 and pocket 4, respectively (Figure S6). Point mutation experiments of P105A and D49A demonstrated that mutation D49A, rather than P105A, significantly diminish the binding of PAC5 to hnRNPA2B1 (Figure 2i).

Taken together, we determined that PAC5 binds to pocket 4 of hnRNPA2B1.
resulting in anti-HBV activity (Figure 2j).

**PAC5 binding hnRNPA2B1 activates TBK1-IRF3 signaling**

TANK-binding kinase 1 (TBK1) is a critical serine/threonine-protein kinase that mediates phosphorylation and nuclear translocation of interferon regulatory factor 3 (IRF3). The TBK1-IRF3 pathway can be activated by hnRNPA2B1 dimers in the cytoplasm. Herein, immunofluorescence results indicated that PAC5 stimulation significantly accelerates the translocation of hnRNPA2B1 from the nucleus into the cytoplasm (Figure 3a), while PAC3, an inactive analogue of PAC5, cannot affect the translocation of hnRNPA2B1 from the nucleus into the cytoplasm (Figure S7). Accordingly, increased accumulation of hnRNPA2B1 dimers was detected in the cytoplasm after the PAC5 treatment (Figure 3b). Further, we found that PAC5 markedly enhanced the phosphorylation of TBK1 and IRF3 (Figure 3c). Interestingly, PAC5 treatment enhanced the association of hnRNPA2B1 with TBK1 as well as IRF3 in HBV-transfected cells (Figure 3d). Thus, PAC5 functions as an activator for hnRNPA2B1 to initiate the TBK1-IRF3 pathway.

To further confirm PAC5 targets hnRNPA2B1 to activate TBK1 in PAC5-mediated suppression of HBV replication, we employed the CRISPR/Cas9 system to knockout hnRNPA2B1 in HepG2.2.15 cells. The result showed that hnRNPA2B1 deficiency diminished the promoting effect of PAC5 on the limitation of HBsAg and HBeAg secretion (Figure 3e). Additionally, hnRNPA2B1 deficiency significantly impaired the activation of IRF3 and TBK1.
upon PAC5 stimulation. It is worth mentioning that the phosphorylation of IRF3 and TBK1 is comparable in the hnRNPA2B1-deficient HepG2.2.15 cells in the presence or absence of PAC5 (Figure 3f). Together, these data indicate that PAC5 binding hnRNPA2B1 to activate the TBK1-IRF3 pathway accounts for the PAC5-induced anti-HBV innate responses.

**PAC5 initiates type I interferon production and hnRNPA2B1 is responsible for PAC5-induced, IFN-mediated anti-HBV response**

PAC5 was demonstrated to activate TBK1-IRF3 signaling. Activation of TBK1-IRF3 can initiate IFN-I productions\(^{15}\). Therefore, we examined the effect of PAC5 on type I interferon by transcriptomics. The result showed a significant difference between NC and PAC5-treated samples (Figure S8a-b). In the PAC5-treated samples, a total of 962 and 568 genes were up-regulated and down-regulated, respectively (Figure S8c). Gene Set Enrichment Analysis (GSEA) found PAC5 activated the processes of RNA splicing, RNA processing, and RNA translation, while superseding the processes of cell adhesion and proliferation (Figure S8d). It is worth noting that PAC5 also activated the type I interferon signaling pathway (Figure S8e). In addition to transcriptome evidence, ELISA analysis revealed that PAC5 treatment significantly increased the production of IFN-\(\beta\) in HBV-infected mice (Figure 4a). In vitro analysis showed that PAC5 enhanced the mRNA levels of IFN-\(\beta\) in HepG2.2.15 cells (Figure 4b). Besides, PAC5 sharply increased the mRNA productions of interferon-stimulated genes (ISG), ISG15 and ISG56, in HepG2.2.15 cells
(Figure 4b). Subsequently, we measured the secreted level of IFN-β in the supernatant and found that PAC5 significantly upregulated IFN-β secretion triggered by HBV infection (Figure 4c).

To further determine whether PAC5-mediated inhibition of HBV replication is type I IFNs signaling dependent, we employed the CRISPR/Cas9 system to knockout IFN-α/β receptor (IFNα/βR) in HepG2.2.15 cells. The result showed that IFNα/βR deficiency diminished the suppressive effect of PAC5 on HBsAg and HBeAg secretion (Figure 4d). Moreover, IFN-β was significantly down-regulated in PAC5-stimulated hnRNPA2B1−/− cells at both transcription and protein expression levels (Figure 4e). The impaired IFN-β production in hnRNPA2B1−/− cells could be rescued by the overexpression of hnRNPA2B1-P105A but not hnRNPA2B1-D49A (Figure 4f). Consistently, PAC5 did not influence HBV-associated antigen secretion in the hnRNPA2B1−/− cells overexpressed with hnRNPA2B1-D49A (Figure 4g). hnRNPA2B1-D49A exhibited limited interaction with TBK1 and IRF3 (Figure 4h), which might lead to the impaired type I interferon activation. Our data further reveal that the residue Asp49 of hnRNPB1 is necessary for PAC5 binding with hnRNPA2B1.

We showed PAC5 binding hnRNPA2B1 activates TBK1-IRF3 signaling and initiates type I interferon production. However, the promotion of autoimmunity targets may be responsible for some of the cell storm factor effects. Herein, we measured the secreted level of TNF-α, IL-6, IL-1β and IL-10 in the supernatant of PAC5-treated animals infected by HBV. The results indicated that PAC5 did
not enhance the secretion of the proinflammatory cytokines (Figure S9).

**PAC5 inhibited SARS-CoV-2 and VSV**

Herein, we assess the function of PAC5 on other viruses, such as pseudotyped SARS-CoV-2 and VSV-G infections. At present, pseudovirus (PsV) has become an ideal tool to analyze cell entry of SARS-CoV-2 without safety concerns and possess the morphological characteristics of replication-competent SARS-CoV-2, with the S protein on the envelope membrane. Here, we first utilized SARS-CoV-2 PsV to perform a series of transduction assays. Results showed that PAC5 exhibited potent inhibitory activity against the infection of SARS-CoV-2 S PsV with the 293T/ACE2 cells (293T cells stably expressing hACE2) in a dose-dependent manner, with an IC$_{50}$ of 0.485 μM (Figure 4i). In addition, inhibitory activities were observed on vesicular stomatitis virus glycoprotein (VSV-G) PsV-infected Vero-E6 cells under the treatment of PAC5 (Figure 4j). The results suggested that PAC5 also stands for a promising strategy for suppressing the broad-spectrum viral replication.

**Discussion**

The most well-known function of type I IFNs is its inherent antiviral activity. Numerous data indicate that type I IFNs serve as a link between the innate immune response to viral infection and the subsequent adaptive immune response. Therefore, new mechanisms inducing and regulating of type IFNs responses has attracted considerable attention. Recently, hnRNPA2B1 has been viewed as an attracting regulatory protein of IFNs.
responses for viral infection. \(^{12,16}\) hnRNPA2/B1, an RNA-binding protein as a member of the hnRNPs family, which plays an important roles in RNA biology and various disease. \(^{10,21}\) However, until now it was unknown whether a small molecule can target hnRNPA2B1.

Herein, we show PAC5 is the first small molecule agonist of hnRNPA2B1 for inhibition of viral infections. Based on an anti-HBV natural sesquiterpenoid phyllanthacidoid A (PA), we designed and synthesized 38 derivatives. Among them, PAC5 showed the most potent anti-HBV activity and no toxicity in cell-based assays. In mice infected with wild-type and drug-resistant HBV, oral administration of PAC5 significantly reduced serum HBsAg and HBeAg status, HBcAg expression, HBV DNA level, and liver inflammatory cell infiltration. PAC5 also showed excellent safety profile in mice. Further, our collective chemical proteomic studies indicate that the principal mechanism of action of PAC5 is due to binding hnRNPA2B1, a drug target previously unknown for inhibition of HBV. Moreover, our data indicate that PAC5 promoted the formation of hnRNPA2B1 homodimer and translocation to the cytoplasm where it activates the TBK1-IRF3 pathway, leading to IFN-\(\beta\) production.

The \(hnRNPA2B1\) gene encodes 2 major protein isoforms, hnRNP A2 and hnRNP B1, through alternative splicing. The hnRNP B1 contains an additional 12-amino acid insertion at the N-terminus. \(^9\) hnRNPA2B1 contains two tandem RNA/DNA-recognition motifs (RRM1 and RRM2) at the N terminus and Gly-rich low complexity (LC) region in C-terminal. In this study, we firstly identified five
drug pockets in the structure of hnRNPA2B1 using Fpocke. The vina results suggested pocket 0 and pocket 4 are relatively favorable pockets for PAC5. Further point mutation experiments confirmed residue Asp49 of pocket 4 is essential for the PAC5 binding with hnRNPA2B1. Therefore, we inferred that the anti-HBV activity of PAC5 is achieved by binding to pocket 4. We observed that the N-pentylamine of PAC5 is deeply embedded in the groove of pocket 4. The results may explain that why the length of N-pentylamine substituent of the norbisabolane sesquiterpene series has a key influence on anti-HBV activity. It suggests the importance of hydrophobic interaction between the N-pentylamine of PAC5 and the groove of pocket 4. Pocket 4 is located in the RRM1 region and overlaps with the RNA binding pocket. In the RNA binding pocket, RRM1 specifically recognizes the AGG motif of 8mer RNA substrates and residue Asp49 is hydrogen-bonded to the nucleotide of the AGG motif \(^9\). The RRM domains are required for hnRNPA2B1 dimer formation \(^{12}\). Taken together, though structural bioinformatics and experiment validation, our study suggested that targeting RRM1 by a small molecule might be an effective strategy to modulate hnRNPA2B1 function.

Given that type I IFN have pleiotropic effects on the HBV life cycle, including epigenetic modification, suppression of transcription of viral RNA, and limitation of encapsulation\(^{22}\), induction of IFN and the innate antiviral response in chronic hepatitis B patients could be expected to reduce not only HBV DNA levels but also the production of other HBV-related antigens (HBsAg and HBeAg). A
recent study identified hnRNPA2B1 as a nuclear receptor for viral DNA critical for the host antiviral responses. As hnRNPA2B1 initiates the activation of the TBK1-IRF3 pathway to turn on type I IFNs\textsuperscript{12}. Our data reveal that the reduction of HBV DNA levels by hnRNPA2B1 agonist treatment was similar to the reductions of serum HBsAg and HBeAg levels in virus-infected mice. Importantly, PAC5 also suppresses other viral replications, such as SARS-CoV-2 and vesicular stomatitis virus (VSV).

In summary, we demonstrated that hnRNPA2B1 is a novel potential broad-spectrum drug target for viral infection. PAC5 targeting this protein inhibit distinct families of viruses that cause significant disease in humans. PAC5 is currently undergoing preclinical development and will be advanced towards human clinical trials.

**Methods**

**Mice and reagents**

C57BL/6J mice were purchased from the Animal Institute of Southern Medical University (Guangzhou, China). All mice were kept in a pathogen-free environment with temperature maintained at 21-23 °C and relative humidity at 50-60%, and underwent a 12 h: 12 h light: dark cycle. All animal experiments in this study were approved by the Welfare and Ethical Committee for Experimental Animal Care of Southern Medical University. Lamivudine (PHR1365) and G 418 disulfate salt (A1720) were purchased from Sigma–Aldrich. Anti-hnRNPA2B1 antibody (sc-32316, diluted 1:500) was
purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-GAPDH antibody (60004-1-Ig, diluted 1:30,000) were purchased from Proteintech (Chicago, IL, USA). TBK1/NAK Rabbit mAb(38066, diluted 1:1,000), Phospho-TBK1/NAK (Ser172) Rabbit mAb (5483, diluted 1:1,000), IRF-3 Rabbit mAb (4302, diluted 1:1,000), Phospho-IRF-3 (Ser396) Rabbit mAb (29047, diluted 1:1,000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-HBV Core Antigen/HBcAg antibody (ab8637, diluted 1:500) was purchased from Abcam (Cambridge, UK).

Cell culture.

HepG2.2.15 were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA) and cultured in DMEM high glucose (GIBCO, Life Technology, Carlsbad, CA, USA) with 10% (v/v) fetal bovine serum (FBS) containing 400 μg/mL of G418, and incubated in a 5% CO2 incubator at 37 °C. The HEK293T cells were purchased from ATCC and cultured in DMEM high glucose supplemented with 10% FBS.

Animal Treatments

The method of establishing HBV infection mouse model is described as previously. Six to eight weeks old C57BL/6 male mice were injected with rAAV8-1.3HBV ayw (1×10^{11} Vg of each/mouse) dissolved in 200 μL PBS via a tail vein. rAAV8-1.3HBV ayw was purchased from Beijing fiveplus Molecular Medicine Institute Company (Beijing, China). Mice were assigned to 5 groups (n=6 per group) and intragastrically gavaged daily with PBS, 3-TC (50mg/kg),
PAC5 (20mg/kg), PAC5 (100mg/kg) or 3TC (50mg/kg) plus PAC5 (50mg/kg), respectively.

The mouse model of viral hepatitis B in lamivudine-resistant was established as previously described. Each male C57BL/6J mouse (6-8 weeks) was hydrodynamically injected with 10 μg PBSK-rtM204I plasmid (dissolved in sterile PBS equivalent to 8% of the mouse body weight) via a tail vein in 6–8 seconds. Mice were divided into 3 groups (n=6 per group) and intragastrically gavaged daily with PBS, 3-TC (50mg/kg), and PAC5 (50mg/kg), respectively. The mice were stopped for intragastric administration of these drugs at week 8 and sacrificed at week 10. Serum HBV DNA, HBsAg, HBeAg levels and hepatic HBcAg expression of virus-infected mice were detected at the indicated time point.

**Determination of HBsAg, HBeAg and HBV DNA**

The levels of HBsAg, HBeAg in culture supernatants or mouse serum were measured by chemiluminescence microparticle immunoassay (CMIA) on an Architect system (Abbott Laboratories, North Chicago, IL, USA). A volume of 60 μL of mouse serum was used to extract HBV DNA, following the manufacturer’s instructions (Qiagen, Hilden, Germany). Then, real-time PCR was performed with Roche Cobas Amplicor PCR assay (Roche Molecular Systems, Branchburg, NJ, USA) using SYBR Green master mix (commercially available assay kit, TOYOBO, Osaka, Japan).

**Histologic and immunohistochemical staining**
Paraffin-embedded liver tissue blocks were cut into 5 μm slices and mounted onto polylysine-charged glass slides. Tissue damage was evaluated by H&E staining. Antigen retrieval was performed in a 100 °C citrate buffer (pH 6.0) for 10 min, then exposed to 3% H$_2$O$_2$ for 10 minutes to inhibit endogenous peroxidase activity. Sections were then incubated with primary antibodies at 4 °C overnight. Immuno-reactivity was detected using the corresponding HRP conjugated secondary antibody and visualized using diaminobenzidine kit (Beyotime Biotechnology, Beijing, China).

**Measurement of serum ALT, AST and inflammatory cytokines**

Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) were measured with commercial kits (Jiancheng Biotech, Nanjing, China), according to the manufacturer’s instructions. The levels of IFN-β in the culture medium or sera were assessed by commercial ELISA kits purchased from Cloud-Clone Corp (Wuhan, Hubei, China).

**Isolation of RNA and quantitative real-time PCR**

Mouse liver total RNA and HepG2.2.15 RNA was extracted using TRIzol reagent (TransGene Biotech, Beijing, China) and then transcribed into cDNA using TranScript All-in-One First-Strand cDNA Synthesis SuperMix (TransGene Biotech), as instructed by the manufacturer. Real-time PCR was performed with 7900HT fast real-time PCR system (Applied Biosystems, San Francisco, CA, USA) using TransStart Tip Green qPCR SuperMix (TransGene Biotech). The expression was normalized to the expression of GAPDH. The
primer sequences used in the experiment are shown below. The primers used were as follows: human IFN-β (forward 5′-AGTGTCAGAAGCTCCTGTGGCAA-
3 and 5′- ATGCGGCGTCCTCCT TCTGGA-3), human hnRNPA2B1 (forward 5′-
-ATTGATGGGAGAGTAGTTGAGCC-3 and reverse 5′-
AATTCCGCCAACAAAACAGCTT-3), human GAPDH (forward 5′-
GTCTCCTCTGACTTCAACAGCG-3 and reverse 5′-
ACCACCTGTGCTGTAGCCA A-3), mouse IFN-β (forward 5′-
AGCTCAAAGAAAGGACGAACA-3 and reverse 5′-
GCCCTGTAGGTGAGGTTGAT-3), mouse GAPDH (forward 5′-
CATCACTGCCACCCAGAAGACTG-3 and reverse 5′-
ATGCCAGTGAGCTTCCCGTTCAG-3 ).

Immunofluorescence

For analysis of the colocalization of PAC5 with hnRNPA2B1, HepG2.2.15 cells were plated on a glass coverslip in a 6-well plate, then incubated with rhodamine-labelled PAC5. For analysis of the subcellar location of hnRNPA2B1, HepG2.2.15 cells were treated with PAC5 or its control analogue for the indicated time point. Cells were fixed with 4% (wt/vol) paraformaldehyde for 15 min and permeabilized with 0.5% (vol/vol) Triton X-100 for another 15 min at room temperature. Subsequently, the cells were blocked in 5% normal goat serum for 1 h at room temperature. Sections were incubated with anti-hnRNPA2B1 (Santa Cruz Biotechnology, diluted 1:100) antibodies overnight at 4°C followed by incubation with Alexa Fluor 488- labeled secondary antibodies.
for 1 h at room temperature and counterstained with 4’,6-diamidino-2-
phenylindole DAPI (Sigma–Aldrich) for 5 min. Sections were mounted with the
Mowiol-based antifading medium (Beyotime Biotechnology) and analyzed with
LSM 880 confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Plasmids and transient transfection.**

The plasmid of Flag-tagged hnRNPA2 (Z4409), Flag-tagged hnRNPB1 (F0171),
HA-tagged IRF3 (Z8111) and HA-tagged TBK1 (S0424) were purchased from
GeneCopoeia (Rockville, MD, USA). hnRNPB1-P105A and hnRNPB1-D49A
plasmid were generated by HNRNPB1-FL plasmid with a P105A and D49A
point mutation. HEK293T cells were seeded in 10-cm dishes and were
transfected with 10 µg of plasmid with PEI (Polysciences, Warrington, PA, USA)
according to the manufacturer’s instruction. HepG2.2.15 cells plated in six-well
plates and were transfected with 3 µg of plasmid with PEI according to the
manufacturer’s instruction.

**Generation of knockout cells with CRISPR/Cas9**

HepG2.2.15 cells were transfected with 2 µg of IFN-α/βRα (Sc-401662) or 3 µg
hnRNPA2B1 (sc-400635-KO-2) CRISPR/Cas9 KO plasmid (Santa Cruz
Biotechnology) using 10 µL UltraCruz transfection reagent (Santa Cruz
Biotechnology), according to the manufacturer’s instructions. 72 h after the
transfection, the expression levels of hnRNPA2B1 in the cells were assessed
by immunoblotting and RT-PCR analysis.

**Protein isolation**
Protein extraction kit ((Beyotime Biotechnology) was used to extract nuclear and cytoplasmic protein from $5 \times 10^6$ HepG2.2.15 cells in accordance with the manufacturer’s instructions. Briefly, cells were dounce homogenized in a solution containing 10 mM HEPES, 50 mM NaCl, 0.5M sucrose, 0.1 mM EDTA, and 0.5% Triton X100. This suspension was spun at 1000 rpm at 4°C to pellet nuclei. The cytoplasmic supernatant was re-spun, and the resulting supernatant was collected. Nuclei were washed in a solution containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA and pellets were then suspended in a solution of 10 mM HEPES, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.1% NP-40. This suspension was vortexed and spun to yield a supernatant containing nuclear proteins. BCA method was used for protein quantification, and then the total protein was boiled for 5 min and stored for preparation.

Immunoprecipitation and Immunoblotting.

HepG2.2.15 or HEK293T cells were lysed in RIPA buffer (50 mM Tris, 150 Mm NaCl, and 1% Nonidet P-40, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 minutes. The lysates were ultracentrifuged at 12,000×g for 15 min at 4°C. The clear supernatants adds 2× SDS loading buffer (containing 25 mmol/l Tris-Cl/SDS, pH 6.8, 4 g SDS, 20 ml glycerol, 1.0 g bromophenol blue, and 3.1 g DTT, add H$_2$O to 100 ml) and boiled at 100°C for 5 min. Protein samples extracted from cells were separated by SDS-PAGE or native PAGE, and then transferred onto polyvinylidene fluoride (PVDF)
membranes (Millipore, Billerica, MA, USA). After blocking with TBS containing 0.05% Tween-20 and 5% BSA for 1 hour at room temperature and incubated with primary antibodies at 4 °C overnight or 2 h at room temperature. Subsequently, incubate the membrane for 50 minutes at room temperature with horseradish peroxidase- (HRP-) conjugated corresponding secondary antibody. For immunoprecipitation, cell lysates were incubated with antibodies (1μg-2μg) or immobilized biotinylated PAC5 (30μM) at 4 °C overnight. Then add 20 μl of protein A/G agarose (Santa Cruz Biotechnology) or 40ul avidin agarose beads (Thermo Fisher) for 4-6 hours at 4 °C. Resuspend immunoprecipitates in 40 μl of Electrophoresis Sample Buffer and continue with electrophoresis and immunoblotting as described above. Finally, measurement of the interest protein was conducted with enhanced chemiluminescence (Thermo Fisher) according to the manufacturer’s protocol.

### Pull-down experiments and Mass spectrometry analysis

HepG2.2.15 (1×10^8) cells were lysed in cell lysis buffer (Biyuntian) containing 1 mM PMSF. Then, the cell lysates were incubated with immobilized biotinylated PAC5(30μM), negative control and blank control at 4° C overnight, followed by incubating with 40 μl avidin agarose beads (Thermo Fisher) for another 4-6 hours. Resuspend immunoprecipitates in 40 μl of Electrophoresis Sample Buffer and boiled at 100°C for 5 min. Total 20 µl Electrophoresis Sample Buffer in each condition was loaded onto SDS-PAGE gels. The proteins were visualized by Coomassie staining. The major bands around the 70-80 kDa
region were cut out separately and subjected to mass spectrometry for protein identification. The gels were digested with trypsin and analyzed using mass spectrometry analysis on an AB Sciex TripleTOF 5600+ system (Novogene, China). All MS/MS spectra were searched against the NCBI database using MASCOT (version 2.3).

**RNA sequencing, Quantification of gene expression, and Gene set enrichment analysis**

RNA sequencing (RNA-Seq) was performed on HepG2.2.15 cells treated with DMSO and PAC5, respectively. Gene expression was inferred from the refined BAM files using feature Counts (v1.6.4)\textsuperscript{25} and reported as transcripts per million (TPM). Differentially expressed genes were analyzed using R language “limma” package (v 3.44.3)\textsuperscript{26}. Gene Set Enrichment Analysis (GSEA) of GO biological processes was performed using the functions “GSEA” in R package clusterProfiler\textsuperscript{27}.

**Pocket identification and molecular docking**

The protein structure (ID: 5HO4) of hnRNPA2B1 is obtained from the PDB database (https://www.rcsb.org/). The potential binding pockets of hnRNPA2B1 were identified by Fpocket (v3) with default setting \textsuperscript{28}. Then the interaction of PAC5 and those pockets of hnRNPA2B1 was predicted by AutoDock Vina (v1.1.2)\textsuperscript{29}. The structures (ID: 5HO4) of hnRNPA2B1 and PAC5 were prepared with AutoDock tools v1.5.6 as suggested in the user guide. The pocket center was set as the docking center. To allow free rotation of the compounds, the
search space was set to $30 \times 30 \times 30$ Å in each axis. The input molecular should be a pdbqt format. All other docking parameters were set to the default values. Each docking is performed by a command that contains space size and three-dimensional coordinates of the docking center. A lower energy score indicated a stronger binding affinity between the ligand and receptor. The binding pose with the lowest energy was selected as the best model for each docking test. The interaction between PAC5 and hnRNPA2B1 was displayed using the PyMOL (v1.7) program (https://pymol.org).

**Statistical analysis**

The experimental data are presented as mean±SEM. For most of the bar graph data, One-way ANOVA was used to analyze the comparisons among multiple groups. In the experiments, differences between two groups were analyzed by Student's t-test. The data were considered statistically significant when the p-value was <0.05.

**Luciferase assay on pseudotyped infection**

To evaluate the inhibitory activity of PAC5 on pseudotyped SARS-CoV-2 or VSV-G infection, 293T/ACE2 or Vero-E6 cells were seeded into 24-well plates at a density of $10^4$ cells per well. After overnight incubation, a series of dilutions of the compound were mixed with an equal volume of pseudovirus (MOI=1), and the mixture was transferred to the cells. 12 hours after infection, the culture medium was replaced with fresh media that contained different concentrations of PAC5. 3 days after the transfection, the cells were lysed with 50 μl of lysis
reagent (Promega) for 15 min. Then, the luciferase activity was quantified by measuring luminescence upon addition of luciferase assay substrate (Promega, USA) using a Synergy HTX (Bio Tek, USA).

Reference


HNRNPA2B1 Is a Mediator of m(6)A-Dependent Nuclear RNA


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School of Pharmaceutical Sciences, Southern Medical University, for performing the pseudovirus neutralization assay. This work was supported by the National Natural Science Foundation of China (31960093, 81973210, 81873872, and 82071781), Innovation team of chronic kidney disease with integrated traditional Chinese and Western Medicine (grant no.: 2019KCXTD014). Program for Innovative Research Team (in Science and Technology) in University of Yunnan Province and Ronald J Quinn AM Academician Workstation (2019IC003).

Author Contributions

Prof. Min Xu contributed to the design of the experiments and conducted the chemical experiments. Mr. Yu Chen and Ms. Jia-Yao Xiang conducted the biological experiments and collected the data. Mr. Hao-Yan Yuan contributed to synthesized PA derivatives. Ms Jia Luo and Miss Jun-Qi Wu contributed to design and synthesis of Probes. Prof. Cheng-Xiang and Dr. Long-Jiao Ge performed toxicity evaluation of PAC5 tests and data analysis. Prof. Ronald J Quinn AM, Prof. Yue-Hai Shen, and Prof. Ya-Ming Li contributed chemical experimental suggestions and revisions. Prof. Yan-Feng and Prof. Hai-Zhou Li contributed biological experimental suggestions. Dr. Hao-Chen contributed blocking experimental suggestions. Prof. Min Xu, Prof. Da-Ming Zuo and Prof. Shao-Xing Dai supervised all phases of the project. They also analyzed data, wrote and revised the manuscript.

Additional Information

The supplementary material available at https://doi.org. Correspondence and requests for materials should be addressed to Prof. Min Xu, Prof. Da-Ming Zuo and Prof. Shao-Xing Dai.
Competing Interests

The authors declare that they have no competing interests.
Figure legends

Figure 1. PAC5 inhibits HBV replication in *vitro* and *vivo*.

a, The chemical structure of PAC5. b, PAC5 inhibited levels of HBsAg and HBeAg in supernatant of HepG2.2.15 cells and HepG2 cells transfected PBSk-rtM2041. c-e, After 2 weeks of intravenous injection of rAAV8-1.3HBV ayw (1×10^11 Vg of each/mouse), C57BL/6 mice were assigned to 5 groups (n=6 per group) and savaged daily with PBS, 3-TC (50mg/kg), PAC5(20mg/kg), PAC5(100mg/kg) or 3-TC (50mg/kg) plus PAC5(50mg/kg). The mice were stopped intragastric administration at week 8 and sacrificed at week 10. c, The serum levels of HBeAg and HBsAg were monitored by chemiluminescence microparticle immunoassay (CMIA), and the HBV DNA was detected by real-time PCR analysis. d, The histologic analysis of livers was performed using H&E staining. Scale bar=100 µm. e-h, C57BL/6 mice were hydrodynamically injected with 10 µg of PBSK-rtM2041 plasmid through the tail vein. Two weeks later, the mice were assigned to 3 groups (n=6 per group) and gavaged daily with PBS, 3-TC (50mg/kg) or PAC5 (50mg/kg). The gavage was stopped at week 8, and the mice were sacrificed at week 10. e, The levels of HBsAg and HBeAg in sera were measured by CMIA at indicated time points. HBV DNA was measured by real-time PCR analysis. f, The histologic analysis of livers was performed using H&E staining. Scale bar=100 µm h, The sera ALT and AST activities were detected at week 10. All data were presented as means ± SD. *p< 0.05, **p<0.01, calculated by unpaired Student's t-test.
Figure 2. hnRNPA2B1 is a drug target of PAC5.

a, The chemical structure of Probes and NC. b, Immobilized biotinylated Probes was incubated with HepG2.2.15 cell lysates, then the PAC5-associated protein was analyzed by mass spectrometry. c, The scatterplot showing the 117 specific proteins that identified in PAC5-treated sample, the top 10 differential expressed proteins were labeled with gene name. d, The top 5 differential expressed proteins with their functions were listed. e, The interaction of PAC5 with the candidate proteins were validated by co-precipitation assay. f, The chemical structure of rhodamine-labeled PAC5. g, The colocalization of PAC5 (red) and hnRNPA2B1 (green) was determined by confocal assay. Scale bar = 5 μm. h, HEK293T cells were transfected with Flag-tagged hnRNPA2 or Flag-tagged hnRNPB1 expressing vectors, respectively. Then, the cell were incubated with biotinylated PAC5, and the interaction of PAC5 with the hnRNPA2B1 isoforms were determined by co-precipitation assay. i, HEK293T cells were transfected with full-length hnRNP B1 (hnRNPB1-FL), hnRNPB1-D49A, or hnRNPB1-P105A, respectively. After incubation with biotinylated PAC5, the cell lysates were immunoprecipitated with streptavidin and examined by anti-Flag antibodies by immunoblot analysis. Similar results were obtained for three independent experiments. j, The predicted binding mode of PAC5 to hnRNPA2B1. (Top panel) The overview of PAC5 binding to the pocket 4 of hnRNPA2B1 using cartoon and surface representation. (Bottom panel) Close-up view of the binding of PAC5 to the pocket 4 of hnRNPA2B1. The pocket 4
Figure 3. hnRNPA2B1 activation is account for the PAC5-induced anti-

HBV innate responses.

a, HepG2.2.15 cells were treated with PAC5 (10 μM) for indicated time points. The intracellular localization of hnRNPA2B1 (green) was determined by confocal microscopy. The nuclei are stained with DAPI (blue). Scale bar=5 μm.

b, HepG2.2.15 cells were treated with PAC5 (10 μM) for the indicated time and the cytoplasmic and nuclear proteins were extracted. The hnRNPA2B1 dimerization was examined by native PAGE. c, HepG2.2.15 cells were treatment with PAC5 (2 μM) as indicated, and the cell lysates were immunoprecipitated anti-hnRNPA2B1 or IgG. The components (IRF3, TBK1, hnRNPA2B1) in the complex were examined by western blotting. Data are representative of three independent experiments with similar results. d, HepG2.2.15 cells were treatment with PAC5 (2 μM) for the indicated time. Phosphorylated (phospho-) and total TBK1, IRF3 were detected by immunoblotting. e, hnRNPA2B1+/− and hnRNPA2B1−/− HepG2.2.15 cells were treatment with or without PAC5 (5 μM) for 24 hours. The productions of HBsAg and HBeAg were detected in the cell culture supernatants via CMIA. f, hnRNPA2B1+/− and hnRNPA2B1−/− HepG2.2.15 cells were treated with or
without PAC5 (2μM) for 9 hours, the phosphorylations of TBK1 and IRF3 were
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SD. **p<0.01, NS, not significant, calculated by unpaired student’s t-test. Data
are representative of three independent experiments with similar results.

Figure 4 PAC5 initiates type I IFN production for anti-HBV response

a, After 2 weeks of intravenous injection of rAAV8-1.3HBV ayw (1×10^{11} Vg of
each/mouse), C57BL/6 mice were assigned to 3 groups (n = 6 per group) and
gavaged daily with PBS, PAC5 (20 mg/kg), or PAC5(100 mg/kg). The mice were
stopped intragastric administration at week 8 and sacrificed at week 10. The
serum IFN-β levels were evaluated by ELISA analysis. b, HepG2.2.15 cells
were treatment with or without PAC5 (2μM) for 9 hours. mRNA levels of the
indicated genes (IFN-β, ISG15, and ISG56) in the HBV-infected cells were
detected by quantitative RT-PCR analysis. c, HepG2.2.15 cells were treatment
with or without PAC5(2μM) for 9 hours. IFN-β concentrations in the culture
supernatant were assayed by ELISA. d, IFN-α/βRα^{+/+} and IFN-α/βRα^{−/−}
HepG2.2.15 cells were treatment with or without PAC5 (5μM) for 24 hours, the
levels of HBsAg and HBeAg in the cell culture supernatants were analyzed via
CMIA. d, hnRNPA2B1^{+/+} and hnRNPA2B1^{−/−} HepG2.2.15 cells were treated with
or without PAC5 (2μM) for 9 hours, the mRNA level of IFN-β was detected by
quantitative RT-PCR analysis. e, hnRNPA2B1^{+/+} and hnRNPA2B1^{−/−}
HepG2.2.15 cells were treated with or without PAC5 (2 μM). The culture
supernatants were collected at 24 hours after PAC5 stimulation and IFN-β
concentrations were assayed by ELISA. Additionally, the cells were collected at 9 hours after PAC5 stimulation, and the mRNA level of IFN-β was detected by quantitative RT-PCR analysis. f-h, hnRNPA2B1+/− HepG2.2.15 cells were transfected with hnRNPA2-FL together with hnRNPB1-FL, hnRNPB1-P105A, or hnRNPB1-D49A, then treated with PAC5 for 24 hours. The amount of IFN-β protein was evaluated via ELISA (f) and the productions of HBsAg and HBeAg were detected by CMIA (g). HEK293T cells were transfected with Flag-tagged hnRNPB1-FL or hnRNPB1-D49A and HA-tagged TBK1 or IRF3 (h). Cell lysates were immunoprecipitated with anti-Flag and examined for HA by immunoblot. All data were presented as means ± SD. **p<0.01, NS, not significant, calculated by unpaired student’s t-test. Data are representative of three independent experiments with similar results. (i,j) PAC5 inhibits the infection of SARS-CoV2 and VSV infection. Antiviral activity of PAC5 against SARS-CoV-2 S PsV infection in HEK293T/ACE2 cells (i). The effect of PAC5 against infection by a VSV-G-pseudotyped in Vero-E6 cells (j).
Figure 1

a. PAC5 (100mg/kg) 3-TC (50 mg/kg)

b. HepG2.2.15 cells

- $IC_{50} = 0.81 \pm 0.9 \mu M$
- $IC_{50} = 5.88 \pm 0.8 \mu M$
- $IC_{50} = 2.11 \pm 0.01 \mu M$
- $IC_{50} = 2.64 \pm 3.8 \mu M$

HepG2 cells (transfected PBSK-rM204I)

- $IC_{50} = 5.88 \pm 0.8 \mu M$
- $IC_{50} = 2.11 \pm 0.01 \mu M$

HBeAg (COI) vs weeks post injection

- Vehicle
- 3-TC (50 mg/kg)
- PAC5 (20 mg/kg)
- PAC5 (100 mg/kg)
- 3-TC (50 mg/kg) + PAC5 (50 mg/kg)

-ALT (U/L) vs weeks post injection

- AST (U/L) vs weeks post injection

HepG2 cells (transfected PBSK-rM204I)

- Vehicle
- 3-TC (50 mg/kg)
- PAC5 (50 mg/kg)
- PAC5 (100 mg/kg)
- 3-TC + PAC5

HepG2 cells (transfected PBSK-rM204I)

- Vehicle
- 3-TC (50 mg/kg)
- PAC5 (50 mg/kg)
- PAC5 (100 mg/kg)
- 3-TC + PAC5

HepG2 cells (transfected PBSK-rM204I)

- Vehicle
- 3-TC (50 mg/kg)
- PAC5 (50 mg/kg)
- PAC5 (100 mg/kg)
- 3-TC + PAC5
Figure 2

a. Probe (PAC5)

b. NC (negative control)

c. 100 kDa → 70 kDa → 55 kDa → 40 kDa

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<td>Ribosomal protein L5</td>
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Figure 2b.

Figure 2d.

Figure 2e.

Figure 2f.

Figure 2g.

Figure 2h.

Figure 2i.

Figure 2j.
Figure 3

a. DAPI, A2B1, Merge

b. PAC5

C. PAC5

D. PAC5

E. HBSAg (COI)

F. Merge A2B1, DAPI, 5 μm, A2B1, GAPDH, dimer, Histone

Control PAC5

A2B1 +/-

A2B1 -/-

**

NS

A2B1 +/

A2B1 -/

Ctrl

PAC5

Ctrl

PAC5

phospho-IRF3

IRF3

phospho-TBK1

TBK1

GAPDH

phospho-IRF3

IRF3

phospho-TBK1

TBK1

GAPDH
Figure 4

a. Concentration of PAC5 (µM)  
   Vehicle PAC5 (20 mg/Kg) PAC5 (100 mg/Kg)  
   IC<sub>50</sub> = 0.485 (µM)  

b. Inhibition of VSV-G PsV (%)  
   Vehicle PAC5  
   IC<sub>50</sub> = 1.716 (µM)  

c. IFN-β (pg/mg)  
   Vehicle PAC5  

---

d. NS  
   Vehicle PAC5  

---

e. IFN-β (pg/mg)  
   Vehicle PAC5  

---

f. IFN-β (pg/mL)  
   Vehicle PAC5  

---

g. HBeAg (COI)  
   Vehicle PAC5  

---

h. B1 Flag B1 D49A Flag IgG  
   IRF3 TBK1 A2B1  

---

i. IC<sub>50</sub> = 0.485 (µM)  
   Inhibition of SARS-2 PsV (%)  

---

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---

Figure 1

PAC5 inhibits HBV replication in vitro and vivo. a, The chemical structure of PAC5. b, PAC5 inhibited levels of HBsAg and HBeAg in supernatant of HepG2.2.15 cells and HepG2 cells transfected PBSk-rTM2041. c-e, After 2 weeks of intravenous injection of rAAV8-1.3HBV ayw (1×10^{11} Vg of each/mouse), C57BL/6
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Figure 4

PAC5 initiates type I IFN production for anti-HBV response. After 2 weeks of intravenous injection of rAAV8-1.3HBV ayw (1×10^{11} Vg of each/mouse), C57BL/6 mice were assigned to 3 groups (n = 6 per group) and gavaged daily with PBS, PAC5 (20 mg/kg), or PAC5 (100 mg/kg). The mice were stopped intragastric administration at week 8 and sacrificed at week 10. The serum IFN-β levels were evaluated by ELISA analysis. HepG2.2.15 cells were treated with or without PAC5 (2 μM) for 9 hours. mRNA levels
of the indicated genes (IFN-β, ISG15, and ISG56) in the HBV-infected cells were detected by quantitative RT-PCR analysis. c, HepG2.2.15 cells were treatment with or without PAC5 (2μM) for 9 hours. IFN-β concentrations in the culture supernatant were assayed by ELISA. d, IFN-α/βRα+/- and IFN-α/βRα/- HepG2.2.15 cells were treatment with or without PAC5 (5μM) for 24 hours, the levels of HBsAg and HBeAg in the cell culture supernatants were analyzed via CMIA. d, hnRNPA2B1+/+ and hnRNPA2B1-/- HepG2.2.15 cells were treated with or without PAC5 (2 μM) for 9 hours, the mRNA level of IFN-β was detected by quantitative RT-PCR analysis. e, hnRNPA2B1+/+ and hnRNPA2B1-/- HepG2.2.15 cells were treated with or without PAC5 (2 μM). The culture supernatants were collected at 24 hours after PAC5 stimulation and IFN-β concentrations were assayed by ELISA. Additionally, the cells were collected at 9 hours after PAC5 stimulation, and the mRNA level of IFN-β was detected by quantitative RT-PCR analysis. f-h, hnRNPA2B1-/- HepG2.2.15 cells were transfected with hnRNPA2-FL together with hnRNBP1-FL, hnRNBP1-P105A, or hnRNBP1-D49A, then treated with PAC5 for 24 hours. The amount of IFN-β protein was evaluated via ELISA (f) and the productions of HBsAg and HBeAg were detected by CMIA (g). HEK293T cells were transfected with Flag-tagged hnRNBP1-FL or hnRNBP1-D49A and HA-tagged TBK1 or IRF3 (h). Cell lysates were immunoprecipitated with anti-Flag and examined for HA by immunoblot. All data were presented as means ± SD. **p<0.01, NS, not significant, calculated by unpaired student’s t-test. Data are representative of three independent experiments with similar results. (i,j) PAC5 inhibits the infection of SARS-CoV2 and VSV infection. Antiviral activity of PAC5 against SARS-CoV-2 S PsV infection in HEK293T/ACE2 cells (i). The effect of PAC5 against infection by a VSV-G-pseudotyped in Vero-E6 cells (j).

**Supplementary Files**

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- NatureSI20210412.pdf
- TableS1.pdf
- TableS2.pdf