

# World Journal of *Gastroenterology*

*World J Gastroenterol* 2021 April 14; 27(14): 1362-1523



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**INDEXING/ABSTRACTING**

The WJG is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, and Scopus. The 2020 edition of Journal Citation Report® cites the 2019 impact factor (IF) for WJG as 3.665; IF without journal self cites: 3.534; 5-year IF: 4.048; Ranking: 35 among 88 journals in gastroenterology and hepatology; and Quartile category: Q2. The WJG's CiteScore for 2019 is 7.1 and Scopus CiteScore rank 2019: Gastroenterology is 17/137.

**RESPONSIBLE EDITORS FOR THIS ISSUE**

Production Editor: Ji-Hong Lin; Production Department Director: Yun-Xiaoqian Wu; Editorial Office Director: Ze-Mao Gong.

**NAME OF JOURNAL**

*World Journal of Gastroenterology*

**ISSN**

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

**LAUNCH DATE**

October 1, 1995

**FREQUENCY**

Weekly

**EDITORS-IN-CHIEF**

Andrzej S Tarnawski, Subrata Ghosh

**EDITORIAL BOARD MEMBERS**

<http://www.wjgnet.com/1007-9327/editorialboard.htm>

**PUBLICATION DATE**

April 14, 2021

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<https://www.wjgnet.com/bpg/gerinfo/242>

**STEPS FOR SUBMITTING MANUSCRIPTS**

<https://www.wjgnet.com/bpg/gerinfo/239>

**ONLINE SUBMISSION**

<https://www.f6publishing.com>



## Intracellular interferon signalling pathways as potential regulators of covalently closed circular DNA in the treatment of chronic hepatitis B

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**Author contributions:** Goh ZY wrote the paper; Ren EC and Ko HL critically edited, revised and finalized the manuscript.

**Conflict-of-interest statement:** Authors declare no conflicts-of-interest for this article.

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**Manuscript source:** Invited manuscript

**Specialty type:** Infectious diseases

**Country/Territory of origin:**

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### Abstract

Infection with the hepatitis B virus (HBV) is still a major global health threat as 250 million people worldwide continue to be chronically infected with the virus. While patients may be treated with nucleoside/nucleotide analogues, this only suppresses HBV titre to sub-detection levels without eliminating the persistent HBV covalently closed circular DNA (cccDNA) genome. As a result, HBV infection cannot be cured, and the virus reactivates when conditions are favorable. Interferons (IFNs) are cytokines known to induce powerful antiviral mechanisms that clear viruses from infected cells. They have been shown to induce cccDNA clearance, but their use in the treatment of HBV infection is limited as HBV-targeting immune cells are exhausted and HBV has evolved multiple mechanisms to evade and suppress IFN signalling. Thus, to fully utilize IFN-mediated intracellular mechanisms to effectively eliminate HBV, instead of direct IFN administration, novel strategies to sustain IFN-mediated anti-cccDNA and antiviral mechanisms need to be developed. This review will consolidate what is known about how IFNs act to achieve its intracellular antiviral effects and highlight the critical interferon-stimulated gene targets and effector mechanisms with potent anti-cccDNA functions. These include cccDNA degradation by APOBECs and cccDNA silencing and transcription repression by epigenetic modifications. In addition, the mechanisms that HBV employs to disrupt IFN signalling will be discussed. Drugs that have been developed or are in the pipeline for components of the IFN signalling pathway and HBV targets that detract IFN signalling mechanisms will also be identified and discussed for utility

Singapore

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0  
 Grade B (Very good): B, B  
 Grade C (Good): C, C  
 Grade D (Fair): D  
 Grade E (Poor): 0

**Received:** January 21, 2021**Peer-review started:** January 21, 2021**First decision:** February 10, 2021**Revised:** February 23, 2021**Accepted:** March 17, 2021**Article in press:** March 17, 2021**Published online:** April 14, 2021**P-Reviewer:** Bowden S, Gu J, Wongkajornsilp A, Zhu WF**S-Editor:** Zhang H**L-Editor:** A**P-Editor:** Liu JH

in the treatment of HBV infections. Together, these will provide useful insights into design strategies that specifically target cccDNA for the eradication of HBV.

**Key Words:** Covalently closed circular DNA; Interferons; *APOBECs*; Epigenetic modification; Hepatitis B virus therapeutics

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**Core Tip:** Hepatitis B virus (HBV) infection remains an incurable disease affecting millions worldwide. Treatment with interferons (IFNs) can eliminate the virus by clearing its persistent genome, covalently closed circular DNA (cccDNA), from infected cells. However, its clinical efficacy is limited as HBV proteins antagonize IFN signalling. Other current therapeutics do not target cccDNA thus cannot eliminate HBV. Therefore, new drugs based on the knowledge of how IFNs cause cccDNA degradation and silencing, as well as insights into how HBV antagonizes IFN-mediated mechanisms are needed. This review summarizes what is known about these processes and highlights drugs and developing therapeutics targeted against them for HBV eradication.

**Citation:** Goh ZY, Ren EC, Ko HL. Intracellular interferon signalling pathways as potential regulators of covalently closed circular DNA in the treatment of chronic hepatitis B. *World J Gastroenterol* 2021; 27(14): 1369-1391

**URL:** <https://www.wjgnet.com/1007-9327/full/v27/i14/1369.htm>

**DOI:** <https://dx.doi.org/10.3748/wjg.v27.i14.1369>

## INTRODUCTION

Chronic hepatitis B (CHB) is a major global health problem affecting about 250 million people worldwide, resulting in 800000 deaths per year<sup>[1]</sup>. It is the main etiological factor and cause of mortality for liver cirrhosis and hepatocellular carcinoma (HCC). Despite the availability of a prophylactic vaccine and antiviral therapies, the very high numbers of chronic carriers for the causative hepatitis B virus (HBV) indicates that current treatment regimens are inadequate in eliminating HBV. The primary reason for this is due to the stability and persistence of its genomic DNA<sup>[2,3]</sup>, the covalently closed circular DNA (cccDNA), even under long-term therapy. This pivotal viral DNA template is solely responsible for generating all HBV transcripts<sup>[4]</sup> and viral proteins. Therefore, sub-detection levels of continually present cccDNA in infected cells and tissues act as template reservoirs for HBV to reactivate and persist long after achieving the treatment endpoint of expressing sub-detection levels of HBV antigens in patient sera. Given its importance in maintaining chronicity of HBV infection, experts in the field have come to a consensus that a curative regime can only be achieved through cccDNA elimination or permanently silencing cccDNA<sup>[5,6]</sup>.

Of the 2 treatment options available, oral administration of nucleoside/nucleotide analogues (NAs) that target the HBV polymerase/reverse transcriptase (pol/RT) are better tolerated in patients to achieve reduced HBV titres<sup>[7]</sup>. However, NAs do not directly target cccDNA and therefore HBV reactivation persists. In contrast, the less tolerated interferon (IFN) treatment has been shown to be directly efficacious in promoting cccDNA clearance or epigenetically silencing cccDNA<sup>[8-10]</sup>. This is not surprising, as IFNs are a group of cytokines released from host cells as natural defence against external stimuli such as viral pathogens<sup>[11]</sup>. IFNs bind to their cognate receptors to elicit an intracellular signalling cascade that activates a set of IFN-stimulated genes (ISGs) with antiviral, immunomodulatory and anti-proliferative functions<sup>[12]</sup>. As such, IFNs are often used to treat viral infections against a range of viruses including HBV<sup>[13]</sup>, hepatitis C virus<sup>[14]</sup>, and West Nile virus<sup>[15]</sup>. Multiple lines of evidence have shown that IFN treatment effectively inhibits cccDNA function and eliminates cccDNA with great potency *in vitro* and in selected CHB patients<sup>[16,17]</sup>. However, the underlying mechanisms of its antiviral functions, especially on cccDNA are poorly understood. This is further compounded by the myriad of adverse effects associated with IFN treatments, such as neuropsychiatric disorders and neutropenia<sup>[18,19]</sup>, greatly

limiting its use in HBV therapy. Moreover, HBV has multiple mechanisms that counteract IFN signalling, dampening the antiviral effect of IFN treatment<sup>[20,21]</sup> to result in poor antiviral response in some patients<sup>[22,23]</sup>. To overcome and enhance the sub-optimal antiviral response from direct IFN administration, a better understanding of the precise mechanisms of how IFNs target cccDNA and how HBV can overcome these is necessary. By targeting host factors and HBV products that modulate intracellular IFN signalling and its antiviral and anti-cccDNA effectors, curative therapies with fewer undesirable pleiotropic adverse effects can be developed. This review will highlight these mechanisms and targets for which therapeutic agents can be and have been developed.

### **cccDNA in HBV life cycle**

cccDNA is a stable non-integrated mini chromosome formed in the nucleus of HBV-infected cells<sup>[24]</sup>. HBV enters cells by engaging the sodium taurocholate cotransporting polypeptide (NTCP) receptor<sup>[25]</sup> and utilizing epidermal growth factor receptor for internalization<sup>[26]</sup>. Following cell entry, HBV releases its core nucleocapsid into the cytoplasm while the genomic material carried within, the partially double-stranded relaxed circular DNA (rcDNA) is transported into the nucleus where it is converted into cccDNA through a series of biochemical steps involving multiple host proteins<sup>[27-29]</sup>. cccDNA contains 4 overlapping open reading frames from which all HBV transcripts are transcribed. These include the 0.7 kb mRNA for the HBx protein, 2.4 kb and 2.1 kb mRNAs that encode three different forms (L, M, and S) of envelope surface proteins (HBs), 3.5 kb pre-Core mRNA that codes for the p22 pre-core protein, a precursor for HBe and another multi-functional 3.5 kb pre-genomic RNA (pgRNA) that is both the template for rcDNA synthesis and also encodes components of the core particle, the core protein (HBc) which forms the viral capsid and the HBV pol/RT<sup>[30]</sup>. pgRNA and pol/RT are encapsidated by HBc to form the core particle, where reverse transcription takes place within to form the full-length (-) strand of rcDNA, followed by the incomplete synthesis of (+) strand to generate the partially double-stranded rcDNA. This newly synthesized rcDNA can then be recycled into the nucleus to generate more cccDNA, maintaining nuclear cccDNA pool and contributing to cccDNA persistence. Alternatively, core particles can be enveloped for secretion to generate progeny virions<sup>[31]</sup>. Since cccDNA plays a central role in this replicative cycle, it is clear to see that HBV would cease to replicate or persist when cccDNA is eliminated.

### **cccDNA copy number and persistence**

cccDNA exists in low copies and persists long after antiviral treatment, accounting for HBV reactivation after cessation of treatment<sup>[32]</sup>. cccDNA is found in every phase of the natural course of HBV infection, even in patients who underwent HBs seroconversion to produce protective anti-HBs antibodies after effective antiviral treatment. Seroconversion or the loss of HBs is an important end goal of HBV therapy as it is associated with positive long-term clinical outcomes such as improvement in liver function and reducing the risk of HCC<sup>[33]</sup>. Surprisingly, there is currently no international standard for HBV cccDNA quantitation, and a universally endorsed HBV cccDNA assay is also lacking. Thus, the kinetics and amount of cccDNA in infected cells are not clearly defined as the cccDNA copy numbers reported merely reflect the assay used in a publication and does not facilitate comparison between studies. Cell culture models suggest that cccDNA persists up to 40 d in infected HepG2 cells, at up to 12.5 copies per infected cell<sup>[31]</sup>. Studies in human liver biopsies show that the copies of cccDNA per cell varies greatly by >5000-fold, from very low copies of 0.03 copies per cell to very high levels of 173.1 copies per cell, and that this is correlated with HBV reactivation status<sup>[3]</sup>. Consistent with the correlation between high cccDNA copies and CHB status, HBe-positive patients have a higher level of cccDNA compared to HBe-negative patients. Moreover, very low cccDNA copies of 1.5 copies per infected hepatocyte is sufficient to cause virus persistence<sup>[34]</sup>. High cccDNA levels has also been shown to be associated with increased risk of liver inflammation<sup>[35]</sup>. Collectively, high cccDNA levels is associated with greater HBV titres, increased risk of HBV reactivation and higher risk of developing HBV-associated liver diseases, emphasizing the need for cccDNA elimination to achieve a complete cure from HBV infection.



## CURRENT HBV TREATMENT STRATEGIES

### Nucleoside/nucleotide analogues

The standard of care for hepatitis B is based on two therapeutic strategies: the use of NAs and IFN- $\alpha$  or its pegylated form (PEG-IFN)<sup>[36,37]</sup>. To date, six NAs have been approved for treatment, including lamivudine, adefovir, entecavir, telbivudine, tenofovir, and tenofovir alafenamide. As NAs are taken orally, they are easy to administer hence promote patient compliance. NAs suppress viral replication by targeting HBV pol/RT activity to disrupt rcDNA synthesis<sup>[38]</sup>. By suppressing HBV load, NAs can alleviate HBV-associated liver diseases, regress fibrosis<sup>[39]</sup> and reduce the risk of developing HCC<sup>[40]</sup>. However, NAs cannot cure HBV infection as loss of virological markers such as HBs is rarely achieved, and seroconversion rates are negligible (Table 1). As a result, HBV reactivation rate is high, with >50% patients showing flares in virological markers (*e.g.* HBV DNA) and biochemical markers for liver damage [*e.g.* alanine aminotransferase (ALT)]. This is often accompanied with irreversible liver decompensation, resulting in death even when re-introduced to lamivudine<sup>[41]</sup>. Thus, to avoid HBV reactivation, CHB patients are often put on long-term (often >10 years) or even life-long NA therapy.

However, long-term administration of NAs leads to drug resistance, with nearly 65% of CHB patients becoming resistant when treated with lamivudine for 5 years<sup>[42]</sup>. This is because NAs target the error-prone HBV pol/RT, generating progeny virions that may carry escape mutations such as rtM204V/I, rtL180M, rtA181T/V, or rtL80V/I in the pol/RT protein sequence, preventing the mutant pol/RT to incorporate NAs into the nascent rcDNA hence escape rcDNA chain termination. As a result, prolonged treatment with NAs allows these “fitter” escape mutants to accumulate, generating drug resistant HBV strains<sup>[43,44]</sup>. As such, new generations of NAs need to be designed to tackle the problem of HBV mutation. Entecavir and tenofovir are such new generation NAs that are currently favoured over first-generation lamivudine and adefovir due to their higher potency and lower occurrence of drug resistance<sup>[45]</sup>. Only time will tell if the lower drug resistance rates indeed hold true for these newer NAs. It is more important to note that, NAs do not target cccDNA as the established cccDNA pool does not require HBV pol/RT for maintenance. Therefore, NAs cannot cure HBV infection.

### IFNs

IFN- $\alpha$  and PEG-IFN are immunomodulators that augment cell-mediated immunity, part of which includes intracellular antiviral activities that can be executed without the aid of immune cells<sup>[46]</sup>. The use of PEG-IFN has superseded standard IFN- $\alpha$  as pegylation improves IFN- $\alpha$  half-life, requiring less frequent dosing<sup>[47]</sup>. More importantly, PEG-IFN- $\alpha$  is more effective in reducing cccDNA levels and also leads to greater rates of ALT normalization with lower HBV reactivation rates (Table 1). When compared to NAs, treatment with IFN and PEG-IFN leads to higher rates of HBe and HBs seroconversion with greater reduction in HBV markers indicative of lower HBV replication rates. Of note, HBs seroconversion is rarely achieved with the use of NAs. Recent clinical studies<sup>[48,49]</sup> have also confirmed that switching from NA therapy to IFN therapy sustains more significant HBe and HBs losses for longer periods, demonstrating the potency of IFNs in the suppression of HBV replication. However, the use of IFNs in the clinical setting is limited due to the need for high dosage and unpredictably variable patient response, which depends on the status of immune cells, HBV titre and type of HBV. It is also less tolerated in patients due to pleiotropic off-target effects from IFN signalling.

At the tissue level, immune tolerance from chronic infection significantly reduces clinical efficacy of IFN treatment. The extracellular arm of the IFN-mediated antiviral response depends on the activation of HBV-specific CD4<sup>+</sup> helper T-cells and CD8<sup>+</sup> cytotoxic T-cells to produce cytokines such as IFN- $\gamma$  and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) that lead to the elimination of HBV-infected cells<sup>[50]</sup>. However, constant exposure to HBV leads to T-cell exhaustion and an immunotolerant environment<sup>[51]</sup>. While IFN- $\gamma$  also induces intracellular antiviral properties, its continued elevation upregulates programmed death-1 (PD-1) immune checkpoint protein on T-cells and also induces its ligand PD-ligand 1 on hepatocytes, leading to immune tolerance as HBV-specific T-cells fail to act on infected cells<sup>[52,53]</sup>. IFN- $\gamma$  also promotes the secretion chemokines from hepatic macrophages that retain CD4<sup>+</sup> T-cells in the liver and induce apoptosis of HBV-specific T-cells, further contributing to HBV evasion of immune clearance. Many other mechanisms of how HBV-specific immune cells' antiviral activities have been augmented in chronic HBV infection have been documented and



**Table 1 Treatment outcomes of nucleoside/nucleotide analogue and interferon therapy**

Ref.	Treatment		% loss of HBV markers				% seroconvert		% normal ALT	% HBV reactivation
	n	Schedule	HBs	HBe	rcDNA	cccDNA	α-HBs	α-HBe		
Reijnders <i>et al</i> <sup>[131]</sup>	132	NA: 16-43 mo	3	42	-	-	-	35	-	56
Song <i>et al</i> <sup>[132]</sup>	98	NA: 6-22 mo	-	35	-	-	-	35	-	49
Jeng <i>et al</i> <sup>[133]</sup>	691	NA: 1-8 yr	6	n.a.	n.a.	-	4	n.a.	-	79
Liem <i>et al</i> <sup>[134]</sup>	45	NA: ≥ 1 yr	2	n.a.	-	-	-	-	-	71
	22	NA: ≥ 2 yr	4	n.a.	-	-	-	-	-	18
Marcellin <i>et al</i> <sup>[135]</sup>	181	NA: 48 wk	0	n.a.	29	-	0	-	44	-
	177	PEG-IFN: 48 wk	4	n.a.	43	-	3	-	59	-
Lau <i>et al</i> <sup>[136]</sup>	272	NA: 48 wk	0	21	22	-	0	19	28	-
	271	PEG-IFN: 48 wk	3	34	32	-	3	32	41	-
van Zonneveld <i>et al</i> <sup>[137]</sup>	165	IFN: 16 wk	23	33	43	-	-	-	62	13
Niederau <i>et al</i> <sup>[138]</sup>	103	IFN: 4-6 mo	10	51	51	-	-	51	50	-
	53	Untreated	0	13	9	-	-	-	9	-
Liu <i>et al</i> <sup>[139]</sup>	38	IFN: 48 wk	-	-	-	47	-	-	Low	-
	38	PEG-IFN: 48 wk	-	-	-	63	-	-	High	-

HBV: Hepatitis B virus; rcDNA: Relaxed circular DNA; cccDNA: Covalently closed circular DNA; ALT: Alanine aminotransferase; NA: Nucleoside/nucleotide analogue; PEG: Pegylated form; IFN: Interferon; n.a.: Not applicable.

reviewed<sup>[54-56]</sup>, together showing very clearly that IFN treatment alone cannot induce effective clearance of HBV, allowing HBV to persist. Indeed, strategies aimed at restoring extracellular anti-HBV immunity in CHB patients such as with anti-PD-1 therapeutics<sup>[57]</sup> and adoptive T-cell therapy<sup>[58]</sup> are being investigated. Since immune tolerance in CHB patients render immune cells non-responsive to IFN therapy, the efficacy of IFN therapy lies heavily on the intracellular arm of IFN-mediated immunity brought about by induction of intracellular antiviral proteins from IFN signaling.

IFNs induce the production of antiviral proteins that specifically disrupt HBV replication and degrade cccDNA. However, HBV has devised multiple strategies that antagonize IFN signalling. These strategies are so effective that even when coupled with NAs, the inability of NAs to reduce the amount of HBV products allows them to continue disrupting IFN signalling, rendering combination therapy efficacious only in selected patients<sup>[59,60]</sup> but redundant in others<sup>[61]</sup>. Multiple factors including type of combination therapy, HBV genotype and level of HBV replication greatly affect IFN treatment efficacy<sup>[62]</sup>. Some studies suggest that sequential NA and IFN therapy is more effective than simultaneous combination therapy<sup>[63]</sup>, a phenomenon which warrants further confirmatory clinical investigation to enhance clinical response rates. HBV itself also significantly affects patient response to IFN therapy, as treatment outcomes are more efficacious in patients carrying the genotype A virus than the genotype D virus<sup>[64]</sup>, and patients are also thrice more responsive to IFN treatment if they carry the genotype B virus than genotype C virus<sup>[65]</sup>. As further proof to the extent in which HBV alters patient sensitivity to IFN treatment, 30.4% of patients with low end-of-treatment HBs levels (< 10 IU/mL) achieve HBs clearance in a 5-year follow-up, in stark contrast to < 10% of patients achieving HBs clearance<sup>[66]</sup> when end-of-treatment HBs levels are ≥ 10 IU/mL. This is further supported by the association of greater PEG-IFN response in patients with HBV DNA levels of < 9 Log<sub>10</sub> copies/mL sera<sup>[67]</sup>. Clearly, the full potential of IFN therapy has yet to be harnessed and directed towards HBV and its cccDNA. To achieve this, a strong understanding of how IFNs target cccDNA specifically and how HBV overcomes such mechanisms is necessary. New therapeutic agents and strategies can then be developed to prevent HBV from interfering with IFN signalling and enhance the anti-HBV and anti-cccDNA activities of IFNs to bring about HBV elimination.

## IFN SIGNALLING

IFNs are key mediators of immunity, comprising a group of cytokines with antiviral properties against a wide range of pathogens. There are 3 types of IFNs (Type I, II, III) (Figure 1) based on the distinct receptors used for signal transduction<sup>[68]</sup>. Amongst them, only type I IFN- $\alpha$ 2a and IFN- $\alpha$ 2b are used clinically in the treatment of HBV infections<sup>[69,70]</sup>, although the mechanisms underlying its clinical efficacy remain poorly understood. IFNs from all 3 types have been shown to possess anti-HBV properties (Table 2), but many have poorer patient tolerability than IFN- $\alpha$ . IFNs also differ in anti-HBV potency, but the underlying reasons for such differences are not clear. At high doses, IFNs non-cytolytically purge or silence cccDNA from infected hepatocytes.

### Activation of ISGs

The antiviral effects of IFNs are achieved through ISGs<sup>[46,68]</sup>, which are induced through the IFN signalling pathway that activates janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling (Figure 1). After IFNs engage their specific IFN receptors, tyrosine kinase 2 (TYK2) transduces the activating signal to result in phosphorylation of STAT1 and STAT2 transcription factors by protein kinase C-delta (PKC- $\delta$ ) necessary for nuclear translocation. The heterodimer interacts with IFN regulatory factor 9 (IRF9) to form the ISG factor 3 (ISGF3) transcription factor complex which translocates into the nucleus by importin-dependent mechanisms. ISGF3 can then induce the expression of antiviral ISGs through IFN-stimulated response elements (ISRE) cis regulatory elements with the consensus sequence "AGTTTCNNTTTCN" in ISG gene promoters. Alternatively, phosphorylated STAT1 may homodimerize to form the IFN-gamma-activated factor (GAF) complex that binds to another cis-element, the IFN-gamma activated site with the "TTCN<sub>2-4</sub>GAA" conserved sequence motif and induce ISG expression in the absence of IRF9<sup>[71]</sup>. As will be further elaborated, each ISG functions differently to bring about the destruction or silencing of cccDNA and other antiviral effects, accounting for pleiotropic effects of IFN treatment. There are a variety of IFN subtypes, each targeting the activation of different sets of ISGs with antiviral properties that may suppress HBV replication (Table 2). By specifically activating these ISGs relevant for cccDNA degradation or silencing, new drugs can be developed that specifically eliminate HBV. In doing so, adverse effects of IFNs that are not well-tolerated in patients may be eliminated as the activation of ISGs that are irrelevant for suppressing HBV replication are by-passed.

### Regulators of IFN signalling

As JAK/STAT signalling is critical for the expression of ISGs, it is tightly regulated by several proteins<sup>[72]</sup>. Suppressor of cytokine signalling (SOCS) 2 and SOCS3 inhibit the IFN signalling cascade by preventing TYK2 activation and hence STAT1 phosphorylation. STAT1 methylation by protein arginine methyltransferase 1 (PRMT1) prevents its binding to the inhibitor protein inhibitor of activated STAT1 (PIAS1), where PIAS1 functions to sequester STAT1 away from its DNA-binding motifs<sup>[73]</sup>. The methylation of STAT1 is in turn negatively regulated by protein phosphatase 2A (PP2A), which inhibits PRMT1 activity<sup>[74]</sup>. These factors can be targeted by HBV to disrupt and dampen the IFN signalling and will be discussed later.

## ANTI-cccDNA MECHANISMS

### Degradation by APOBECs

Many of the IFN subtypes with anti-HBV properties induce APOBEC family genes<sup>[75]</sup>, which are cytidine deaminases that perform RNA editing<sup>[76]</sup>. They have also been well-documented to act directly on cccDNA to result in degradation. IFN- $\alpha$ , which is used in clinical therapy of CHB, has been shown to reduce the expression of cccDNA, HBV RNA, HBs and HBe in primary human hepatocytes (PHH) and HepRG liver cells. The IFN- $\alpha$  mediated loss of cccDNA was found to last up to 15 d<sup>[8]</sup>. Induced expression of APOBECs can be seen after IFN treatment in liver biopsies of hepatitis B patients, and in HBV-infected chimpanzees<sup>[77]</sup>. The importance of APOBECs in mediating cccDNA clearance is supported by the fact that the expression of APOBECs correlates with clinical response to IFN.

Besides IFN- $\alpha$  and IFN- $\gamma$ , other pathways induced by pro-inflammatory cytokines including TNF- $\alpha$  and lymphotoxin- $\beta$  (LT- $\beta$ ) have also been shown to induce APOBECs and affect the stability of cccDNA. In agreement with its anti-cccDNA functions<sup>[78]</sup>, APOBECs induced through LT- $\beta$  signalling with LT $\beta$ R agonist antibody was shown to

Table 2 Interferons with anti-hepatitis B virus properties

Types of Interferon		Effect on HBV				Remarks	Antiviral ISGs	Ref.
		cccDNA	rcDNA	HBs	HBe			
I	IFN- $\alpha$ 2a/PEG-IFN- $\alpha$ 2a	↓	↓	↓	↓	Clinical anti-HBV agent	APOBEC3A <sup>1</sup> ,APOBEC3F <sup>1</sup> , APOBEC3G <sup>1</sup> ,STAT1 <sup>1</sup> ,ISG20 <sup>1</sup> , TRIM38, MX1, <i>etc.</i>	[140-142]
	IFN- $\alpha$ 2b/PEG-IFN- $\alpha$ 2b	↓	↓	↓	↓	Clinical anti-HBV agent	IFITM1, IFITM3, TRIM14, RNASEL <i>etc.</i>	[143-145]
	IFN- $\alpha$ 14	↓	-	↓	↓	Activates IFN- $\alpha$ and IFN- $\gamma$ signalling	GBP4, GBP5	[146]
	IFN- $\beta$ /PEG-IFN- $\beta$	↓	↓	↓	↓	-	MX1, CXCL10	[147]
	IFN- $\omega$	-	↓	↓	↓	-	IRF1, IRF9, ISG15, OAS	[148-150]
II	IFN- $\gamma$	-	-	-	↓	-	APOBEC3G <sup>1</sup> , OAS, IDO	[151-153]
III	IFN- $\lambda$ 1a	-	↓	↓	↓	Increased HBe seroconversion	PKR, OAS	[154,155]
	IFN- $\lambda$ 2	↓	↓	-	↓	-	APOBEC3A <sup>1</sup> ,APOBEC3B <sup>1</sup> , APOBEC3G <sup>1</sup> , MX1, OAS	[156]
	IFN- $\lambda$ 3	-	↓	↓	↓	Increased JAK/STAT signalling	APOBEC3G <sup>1</sup> ,IRF9 <sup>1</sup> , IRF7, MX1, OAS, ISG15 <i>etc.</i>	[157-159]

↓: Reduced; -: Not determined.

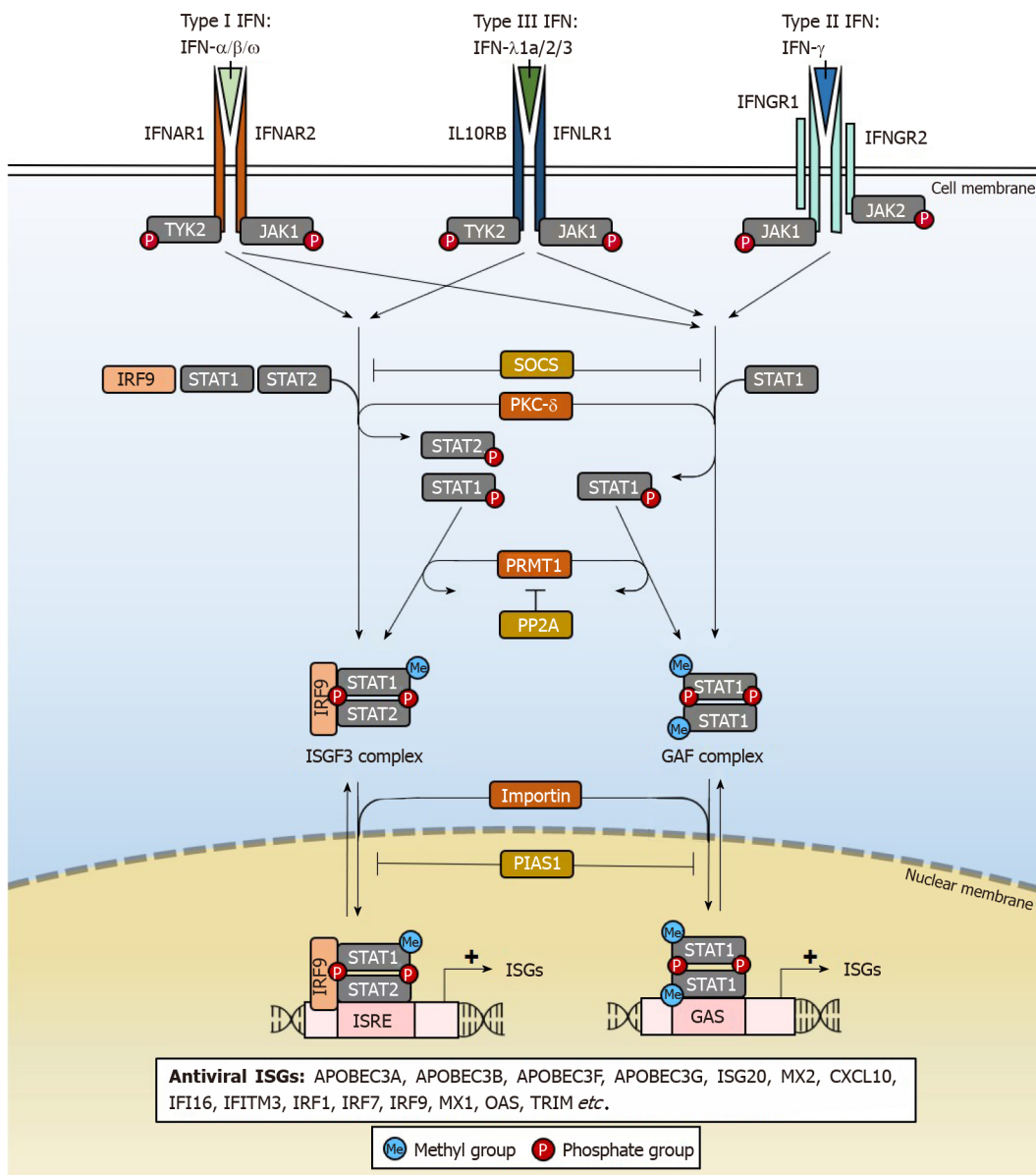
<sup>1</sup>Important for cccDNA elimination or silencing. HBV: Hepatitis B virus; rcDNA: Relaxed circular DNA; cccDNA: Covalently closed circular DNA; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; ISGs: Interferon-stimulated genes; PEG: Pegylated form; IFN: Interferon.

clear 90% of cccDNA in HBV-infected cultured liver cells with good *in vivo* tolerability<sup>[8]</sup>. As the clinical dose of IFN- $\alpha$  used to achieve cccDNA clearance is relatively high, this suggests that inducing expression of APOBECs through LT- $\beta$  signalling is an alternative means for cccDNA clearance with potentially fewer adverse effects. While its clinical efficacy and safety remain to be studied, upregulation of natural ligands for LT $\beta$ R in HBV-infected liver tissues have been reported<sup>[79]</sup>. IFN- $\gamma$  and TNF- $\alpha$  also exhibit anti-cccDNA effects to degrade cccDNA<sup>[80]</sup> levels without causing cell death in HBV-infected chimpanzees, PHH and cultured liver cells.

Following IFN receptor or LT $\beta$ R activation, the upregulated IFN- $\alpha$  induced APOBEC3A (A3A) and LT $\beta$ R activation-induced APOBEC3B (A3B) are brought to cccDNA by HBc (Figure 2). Similarly, IFN- $\gamma$  induced the expression of both A3A and A3B while TNF- $\alpha$  induced the expression of A3B only, whereas knockdown of A3A and A3B abrogated the antiviral effects of IFN- $\gamma$  and TNF- $\alpha$ . HBc amino acids 77-149 are crucial for this interaction, which brings the APOBECs into close proximity with cccDNA for deamination<sup>[8]</sup>. cccDNA deamination generates apurinic/aprimidinic (AP) sites that are recognized by endonucleases<sup>[81]</sup>, which ultimately degrade cccDNA. The dependence on AP sites for cccDNA degradation was confirmed by reduced intact DNA amount when DNA extracted from IFN- $\gamma$  or TNF- $\alpha$  treated cells were digested with recombinant APE1, an AP endonuclease which specifically recognizes and cleaves AP sites<sup>[9]</sup>. Surprisingly, knockdown of APE1 in IFN- $\gamma$  and TNF- $\alpha$  treated HBV infected cells did not show a reduction in cccDNA clearance, suggesting the redundancy of endonucleases in clearing cccDNA. While AP sites can be repaired by the host cells' DNA repair machinery, this is not observed with IFN-mediated AP site formation in cccDNA. This is most likely due to the concurrent downregulation of the base excision repair enzymes such as thymine DNA glycosylase and Nei-like DNA glycosylase after IFN treatment<sup>[77]</sup>. These studies indicate that A3A and A3B are key proteins responsible for IFN and LT $\beta$ R mediated cccDNA clearance. APOBECs also have other important roles in clearing HBV cccDNA. APOBEC3F (A3F) and APOBEC3G (A3G) were also found to have anti-HBV properties<sup>[82]</sup>. A3G for example inhibits pgRNA packaging to reduce virion formation<sup>[83]</sup>. The mechanism of action for A3F has not been well-characterized.

### Epigenetic silencing and transcriptional repression

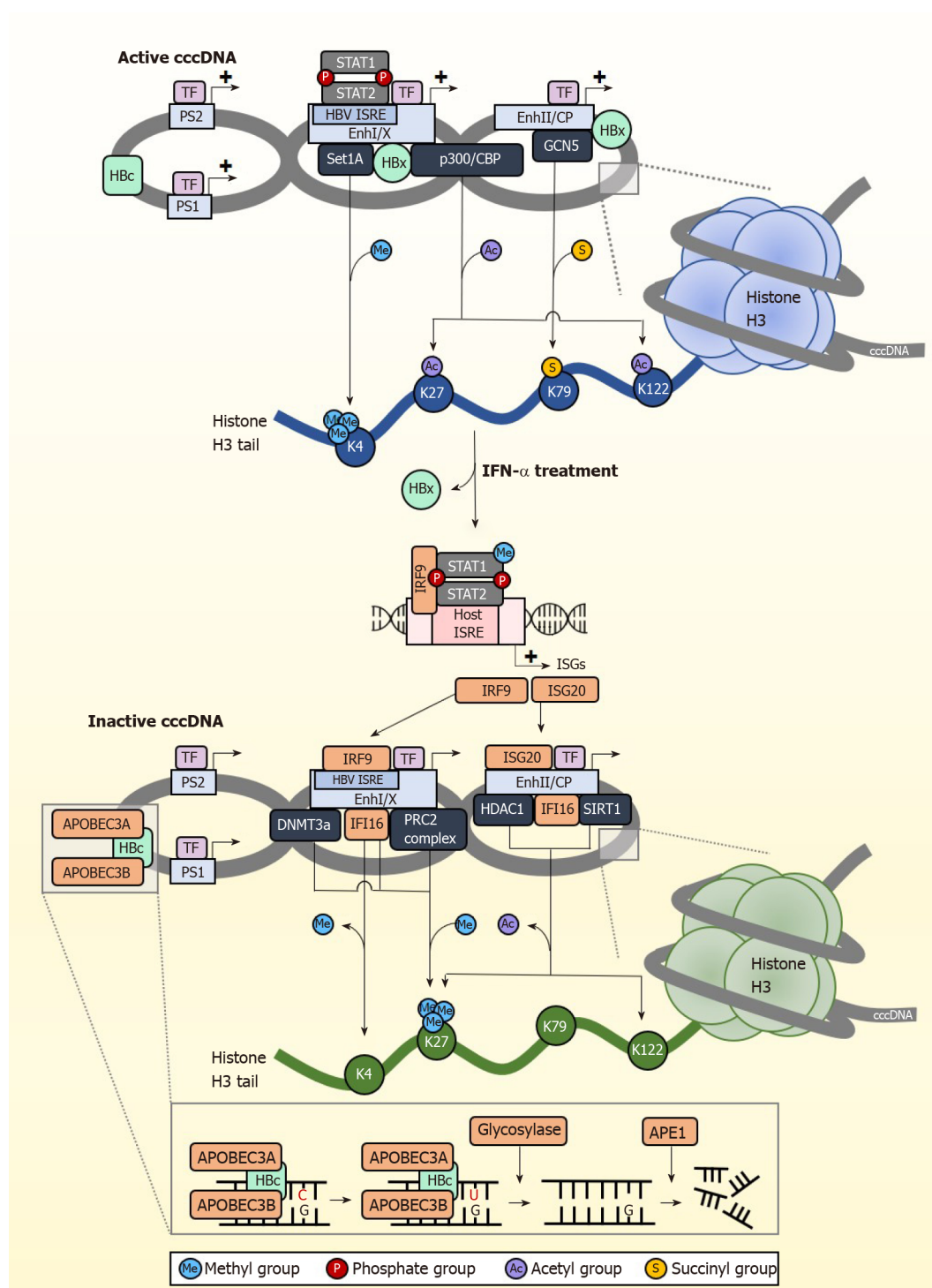
Apart from eliminating cccDNA, permanently silencing cccDNA is another strategy for the development of anti-HBV therapy. Several studies have shown that IFNs control the epigenetic silencing of cccDNA<sup>[84,85]</sup>, spurring growing interest in



**Figure 1 Activation of antiviral interferon-stimulated genes by different interferon subtypes.** Different subtypes of interferons (IFNs) bind to their cognate receptors to trigger IFN signalling pathways. The activate janus kinase/signal transducers and activators of transcription-dependent signalling results in the formation of transcription complexes that induce the expression several IFN-stimulated response elements-dependent and gamma activated site-dependent antiviral IFN-stimulated genes (ISGs), which target covalently closed circular DNA (cccDNA) stability and function through a variety of mechanisms. The potency of IFN subtype for cccDNA degradation or silencing is dependent on the types and number of ISGs induced. IFN: Interferon; TYK2: Tyrosine kinase 2; SOCS: Suppressor of cytokine signalling; PKC-δ: Protein kinase C-delta; IRF: Interferon regulatory factor; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; ISGF: Interferon-stimulated gene factor; GAF: Gamma-activated factor; ISGs: Interferon-stimulated genes; ISRE: Interferon-stimulated response elements; GAS: Gamma activated site.

controlling cccDNA transcriptional activity through epigenetic modifications<sup>[86]</sup>. HBV cccDNA exists as episomal chromatin wound around cellular histones that undergo post-translational modifications (PTMs)<sup>[87]</sup>, altering cccDNA chromatin compaction hence accessibility to transcription regulators (Figure 2). Some of the histone marks associated with active cccDNA transcription include H3K4me3, H3K27ac, H3K122ac, and repressive PTMs include H3K27me3. Of note, the distribution of these histone marks varies across different types of HBV-producing samples<sup>[10]</sup>. In the HepG2-NTCP cell model, histone PTM enrichment is mainly confined to the pre-core/core promoter (CP) region whereas in PHH, they are found throughout the genome with the greatest modifications at the X and pre-S1 promoter regions. In contrast, HBV-infected liver tissues have few PTMs at the CP but accumulate them near pre-S2 and the X promoter regions. The reason and basis for this variability is at present unclear.

cccDNA transcription activity is heavily influenced by the state of epigenetic modification by host cellular factors and PTMs. The cccDNA mini chromosome is



**Figure 2 Interferon treatment silences covalently closed circular DNA transcription and recruits APOBECs to actively degrade covalently closed circular DNA.** Covalently closed circular DNA (cccDNA) exists as an episomal mini chromosome that is epigenetically modified to support active transcription. Interferon (IFN)- $\alpha$  treatment results in the recruitment of histone modifying complexes that remove activating transcription post-translational modifications (PTMs), and add repressive PTMs that silence cccDNA function. These complexes can be recruited by IFN-stimulated genes (ISGs) such as IFI16. ISGs may also bind directly to cccDNA to repress transcription. Other ISGs such as APOBECs induced by IFN signalling are also recruited by HBc, generating AP sites which lead to cccDNA degradation. HBV: Hepatitis B virus; IFN: Interferon; IRF: Interferon regulatory factor; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; ISGs: Interferon-stimulated genes; ISRE: Interferon-stimulated response elements; cccDNA: Covalently closed circular DNA.

heavily modified by various activating PTMs under normal circumstances (Figure 2). H3K4 methyltransferase Set domain containing 1A (Set1A) is recruited to cccDNA promoter sites by HBx, depositing the activating PTM H3K4me3 to drive active transcription<sup>[88]</sup>. By modulating the expression of Set1A, the relative expression of H3K4me3 can be fine-tuned. IFN treatment disrupts cccDNA transcription activity by



downregulating these epigenetic PTMs that support transcription. In studies using PHH, IFN- $\alpha$  specifically reduced trimethylation of H3K4 and acetylation of H3K27 and H3K122 on cccDNA chromatin to inhibit transcription of HBV RNA, but had negligible effect on epigenetic modification for the control promoters of ACTB and Nanog in the host genome<sup>[40]</sup>.

IFNs also result in cccDNA transcriptional repression through active recruitment of complexes that confer transcription inhibitory epigenetic modifications. The repressive H3K27me3 PTM is induced by IFN- $\alpha$  through increased binding of polycomb repressive complex 2 (PRC2) to cccDNA<sup>[84]</sup>. The importance of H3K27me3 in inhibiting cccDNA transcription was also confirmed in a separate study showing that upregulation of DNA methyltransferase 3a hypermethylates HBV cccDNA to repress transcription<sup>[89]</sup>. IFNs have also been recently shown to inhibit succinylation of cccDNA histones, adding on to the transcriptionally repressed state brought about by hypoacetylation and/or methylation<sup>[90]</sup>. This involves the succinylation of H3K79 by GCN5 histone succinyltransferase (also known as lysine acetyltransferase 2A), which corresponds to higher HBV replication. They further showed that low levels of succinylated H3K79 from GCN5-specific knockdown resulted in significantly reduced cccDNA levels, and that expression of GCN5 and cccDNA correlate well in HBV-infected individuals. More importantly, IFN- $\alpha$  treatment could overcome the effects of overexpressed GCN5 to reduce the levels of succinylated cccDNA, indicating that IFNs act upstream to control GCN5 function and repress cccDNA transcription, the specific mechanism of which remains to be elucidated. Taken together, IFNs epigenetically silence cccDNA function through the recruitment of epigenetic factor complexes that add repressive PTMs or remove activating PTMs so that cccDNA enters a transcriptionally repressed chromatin structural state. Importantly, IFNs can also do so by inducing ISGs that bring about both the removal of activating PTMs and the addition of suppressive PTMs. For example, IFI16 reduces cccDNA transcription activity<sup>[91]</sup> by recruiting histone deacetylase 1 (HDAC1) and Sirtuin 1 (SIRT1) to increase repressive H3K27me3 PTMs on cccDNA, and concurrently impairs the recruitment of p300/CBP to prevent the addition of activating PTMs on cccDNA.

Interestingly, cccDNA also carries the ISRE cis-element, and this is critical for establishing IFN-mediated epigenetic changes on cccDNA. The HBV ISRE is located at the enhancer I/X promoter region<sup>[92,93]</sup>, and is recognized by the ISGF3 complex and the ISGs IRF1 and IRF7. In transcriptionally active cccDNA, the HBV ISRE is bound by phosphorylated and unphosphorylated STAT1 and STAT2 transcription factors to active cccDNA (Figure 2). IFN treatment induces redistribution of the STAT proteins from the HBV ISRE towards the IFN signalling pathway, resulting in antiviral effects against HBV cccDNA by the upregulation of cccDNA-targeting ISGs. One of these ISGs is IRF9, which binds directly to the HBV ISRE element to suppress cccDNA transcription<sup>[94]</sup>. When the HBV ISRE is mutated, loss of IRF9 binding was shown to abrogate IFN-induced suppression of cccDNA transcription. This is clinically significant, as mutations in the HBV ISRE affects CHB patient response to IFN treatment<sup>[95]</sup> to render IFN treatment less effective. In addition, the HBV ISRE sequence is HBV genotype dependent, thus its sequence-dependent functionality partially accounts for differences in patient responder rates between carriers of HBV genotypes B and C<sup>[96]</sup>. Another ISG, ISG20, also directly inhibits transcription from cccDNA by direct binding to the enhancer II/CP region<sup>[97]</sup>. Higher ISG20 expression level also correlates to better response to IFN- $\alpha$  treatment in CHB patients<sup>[98]</sup> and viral clearance in HBV-infected chimpanzees<sup>[99]</sup>. With the withdrawal of activating transcription factors from cccDNA and binding of specific transcription repressors induced by IFN treatment, cccDNA transcription is further suppressed by epigenetic modifications of histones such as histone hypoacetylation which occurs through the recruitment of the HDAC1 and SIRT1, and hypermethylated by the PRC2 complex. Thus, in addition to epigenetic silencing of cccDNA, IFNs also suppress cccDNA transcription activity by generating IFN-induced transcription repressors specific to cccDNA.

### ***Inhibition of cccDNA synthesis***

IFNs are also known to directly inhibit cccDNA synthesis. The antiviral ISG myxovirus resistance protein 2 has been shown to reduce cccDNA formation when overexpressed<sup>[100]</sup>. Its specific knockdown abrogates the loss of cccDNA induced by IFN- $\alpha$ , providing confirmation for its role in IFN- $\alpha$  induced reduction in cccDNA levels. It has been proposed that this occurs through inhibiting cccDNA synthesis from rcDNA, as well as from downregulated HBV transcripts.



## HBV DISRUPTION OF IFN SIGNALLING

HBV is not without its defences. It generates multiple factors that dampen the IFN signalling pathway. Inter-individual variability in the balance of these against antiviral IFN-mediated mechanisms could explain why CHB patients do not respond equally well to IFN-treatment. By understanding how HBV proteins antagonize IFN action, therapeutic approaches that interrupt the anti-HBV functions of IFNs can be developed to improve the response and efficacy of IFN treatments.

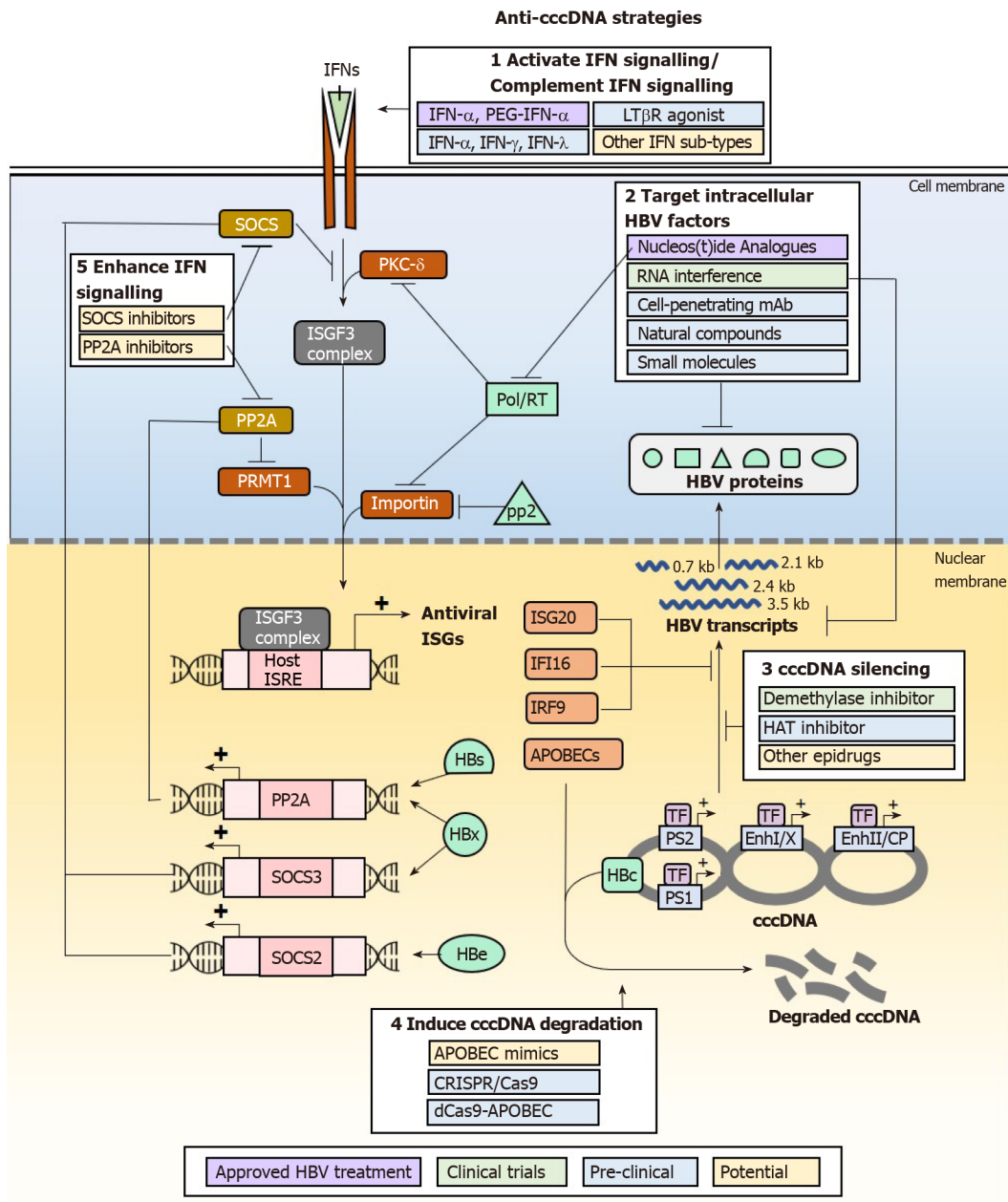
HBV disrupts the IFN signalling cascade at various steps. HBx<sup>[21]</sup> and HBe<sup>[20]</sup> downregulate the expression of IFN receptors, IFNAR1 and IL10RB, disrupting the initiation of type I and type III IFN signalling (Figure 3). HBe and HBx achieve this by upregulating SOCS2 and SOCS3 respectively, suppressing the IFN signalling cascade and inhibiting TYK2 activation (Figure 1). As SOCS2 also prevents STAT1 phosphorylation, STAT1 is prevented from entering the nucleus to induce ISG expression. Since SOCS2 and SOCS3 are the most direct upstream inhibitors of the IFN signaling pathway, their increased expression significantly attenuates IFN-dependent transcription of ISGs to compromise the therapeutic efficacy of IFNs. It was found that overexpression of HBe alone leads to 6-fold reduction in phosphorylated STAT1 nuclear translocation, hence downregulating protein kinase R (PKR) and oligoadenylate synthetase (OAS) gene expression by 50%<sup>[20]</sup>. Similarly, HBx overexpression alone doubled SOCS3 expression and increased PP2A expression by 5-fold, significantly reducing PKR and OAS expression by 3-fold<sup>[101]</sup>. It is thus clear to see, that the combined effects of expressing HBx and HBe in infected cells, especially in cells with high viral titre and virological markers, will compromise the clinical efficacy of direct IFN therapy. Other HBV proteins act further down the IFN signalling cascade to prevent STAT1 nuclear translocation, further disrupting ISG expression. HBV pol/RT inhibits PKC- $\delta$ , preventing STAT1 phosphorylation and competitively binding to importin<sup>[102,103]</sup>. HBV pol/RT also significantly reduces the induction of IRF9 critical for the generation of ISGs through formation of the ISGF3 complex. p22 also binds importin<sup>[104]</sup> to prevent STAT1 nuclear translocation. In the nucleus, HBx and HBs upregulate PP2A, which inhibits PRMT1 hence reduces STAT1 methylation, enabling PIAS1 to sequester phosphorylated STAT1 away from its DNA-binding elements *in vitro* and in liver tissues from HBV infected patients<sup>[105]</sup>. As HBV proteins specifically target multiple regulatory elements of IFN signalling to block ISG expression, the expression of anti-cccDNA and antiviral ISGs would therefore be significantly reduced in the presence of HBV, rendering the effort to eliminate HBV by direct IFN administration futile. Therefore, to circumvent HBV mechanisms that antagonize IFN signalling, therapies aimed at targeting HBV molecules and components of the IFN-signalling pathway would greatly enhance the efficacy of IFN treatment for the elimination and suppression of cccDNA and HBV.

## THERAPEUTIC AGENTS AND STRATEGIES AGAINST cccDNA

IFNs may eliminate and suppress cccDNA formation for a therapeutic cure of HBV infections, but IFN therapy has not been particularly efficacious due to the adverse effects from pleiotropic off-target effects and the existence of multiple HBV mechanisms that antagonize the IFN signalling pathway and downstream effector functions. As these greatly limit the clinical use of IFNs for treating HBV infections, alternative approaches that specifically enhance the anti-HBV effects of IFNs and suppress the antagonistic effects of HBV proteins can be developed to achieve cccDNA elimination and suppression without adverse effects from IFN signalling. Multiple classes of drugs that are in development or have been developed, that act on such targets may have the beneficial effect by downregulating HBV cccDNA (Figure 3). They act by modulating the functions of IFN signalling pathway components or HBV proteins that antagonize IFN signalling and its anti-cccDNA effector functions. Of note, as HBV disruption of IFN response is multi-factorial, combination therapy may be necessary to achieve cccDNA elimination for a true HBV cure.

### Inducers of APOBECs

Perhaps the most obvious strategy to eliminate cccDNA is to enhance and sustain the expression of APOBECs which actively result in cccDNA degradation. While APOBEC mimics are not yet available, APOBEC expression can be induced through multiple pathways including activation of IFN signalling using other IFN sub-types such as IFN- $\gamma$  and IFN- $\lambda$ , and alternative pathways involving TNF- $\alpha$  and LT $\beta$ R activation.



**Figure 3 Anti-covalently closed circular DNA strategies and antagonistic hepatitis B virus proteins that modulate the interferon signalling pathway.** Hepatitis B virus (HBV) has developed multiple mechanisms that target different parts of the intracellular interferon (IFN) signalling pathway to avert elimination by IFN treatment. Therapeutic agents that counter HBV anti-IFN signalling activities or enhance the strength of host cell IFN signalling may be beneficial to increase the efficiency of IFN treatment for the elimination or silencing of covalently closed circular DNA. HBV: Hepatitis B virus; IFN: Interferon; PEG: Pegylated form; SOCS: Suppressor of cytokine signalling; PKC- $\delta$ : Protein kinase C-delta; IRF: Interferon regulatory factor; JAK: Activate janus kinase; STAT: Signal transducers and activators of transcription; Pol/RT: Polymerase/reverse transcriptase; TNF: Tumour necrosis factor; ISGs: Interferon-stimulated genes; ISRE: Interferon-stimulated response elements; cccDNA: Covalently closed circular DNA.

IFN- $\lambda$ 3 was found to be specifically upregulated in patients treated with adefovir or tenofovir, and further shown in cell culture models to be effective in reducing HBs by inducing ISGs<sup>[106]</sup>. Studies in HepaRG differentiated hepatocytes also show that IFN- $\beta$ , IFN- $\lambda$ 1 and IFN- $\lambda$ 2 induce longer-lasting APOBEC expression than IFN- $\alpha$ 2, and are just as efficient in mediating cccDNA degradation<sup>[107]</sup>. IFN- $\gamma$  and TNF- $\alpha$  have also been shown to upregulate APOBEC expression. In particular, the utility of IFN- $\gamma$  in the treatment of HBV infections can be explored as it is currently used clinically for the treatment of hepatic fibrosis<sup>[108]</sup>. An LT $\beta$ R agonist antibody has already been developed and was shown to be safe in mice at lower dosing requirements than clinical IFN- $\alpha$ . This could be further developed and tested in clinical settings for efficacy and safety in the treatment of HBV infections.

The IFN response may also be strengthened by reducing the action of negative regulators of the IFN signalling pathway, primarily by acting on SOCS2 or SOCS3 and

PP2A. This directly inhibits the degradation of IFN signaling, allowing ISGF3 and GAF complexes to form hence carry out transcription of ISGs. Thiazolidinediones such as pioglitazone and rosiglitazone are known to reduce the expression of SOCS proteins<sup>[109]</sup>, and consequently would prevent the loss of IFN signalling transduction hence upregulate APOBEC expression in the presence of appropriate IFNs. They are currently used in the treatment of type II diabetes, thus may be readily re-purposed for the treatment of HBV infections to reduce cccDNA levels. The first-in-class small molecule inhibitor of PP2A, LB-100, would theoretically achieve similar effects of inducing APOBEC expression. It was found to be well-tolerated in patients with solid tumours in phase I clinical trials<sup>[110]</sup>.

### **Epidrugs**

As cccDNA function is highly dependent on its epigenetic PTM status, “epidrugs” are also being investigated for their efficacy in silencing cccDNA function. HAT inhibitor C646, which is widely used in cancer studies, has been shown to reduce H3K27ac and H3K122ac thus silence cccDNA for reduced HBV transcription<sup>[110]</sup>. The pro-drug GS-5801 (Gilead), which specifically inhibits KDM5 (lysine demethylase 5) has been shown to suppress cccDNA transcription by removing H3K4me3<sup>[111]</sup>. Given the importance of other epigenetic modifiers such as GCN5 and p300/CBP for cccDNA transcriptional activation, and SIRT1, HDAC1 for transcriptional repression and silencing, other unexplored epidrugs that inhibit the transcription activation group or activate the transcription repression and silencing group may also be tested for efficacy in directly silencing cccDNA. While epidrugs directly silence cccDNA *in vitro*, their greatest challenge in utility as an anti-HBV therapeutic depends on their ability to specifically target cccDNA in infected cells while sparing host genome to avoid carcinogenesis and toxicity<sup>[112,113]</sup>. In addition, due to compromised functionality of HBV-infected livers, many drugs that have safe profiles in the treatment of non-liver diseases are contraindicated in CHB. For example, the Food and Drug Administration-approved DNMT inhibitor, 5-azacytidine commonly used in the treatment of hematologic malignancies is contraindicated in patients with advanced liver cancer to control HBV infection due to hepatotoxicity<sup>[114]</sup>. Thus, epidrugs need to be carefully evaluated for safety and suitability in the treatment of HBV infections.

### **Targeted degradation of HBV products**

To avoid drug-induced toxicity, approaches that directly target HBV products (cccDNA, RNA and proteins) are being developed. One such approach is to directly degrade cccDNA using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats)/CRISPR-associated protein 9) DNA editing machinery, where guide RNA-directed gene editing of cccDNA specifically results in its erroneous repair by non-homologous end-joining which cleaves and/or mutates cccDNA. This has been shown to effectively reduce cccDNA copies and is followed by concomitant clearance of HBs, HBc and HBe expression in mice with humanized liver<sup>[115,116]</sup> and multiple human liver cell lines<sup>[117,118]</sup>. While the cccDNA-targeting system is being developed and improved for clinical use, a major concern for this strategy is the generation of off-target double-strand breaks in the host genome that may facilitate HBV genomic integration to cause liver cancer. A suggested solution for this is to tether impaired Cas9 to APOBECs so that cccDNA may be mutated by base-changes instead of strand-breaks<sup>[119]</sup>, highlighting again the importance of APOBECs in the role of targeted cccDNA degradation.

Another approach that has potentially fewer off-target effects is to specifically destroy HBV proteins that disrupt the IFN signalling pathway. Cell-penetrating monoclonal antibodies (mAb) have been developed for such purpose. For example, the genetically engineered antibody 9D11-Tat, formed by fusing a cell-penetrating peptide on the C-terminus of the heavy chain of a mAb specific to HBx, can target HBx for proteosomal degradation<sup>[120]</sup>. Alternatively, the transcripts for HBV proteins may be targeted for degradation by small interfering RNA (siRNA) duplexes<sup>[121]</sup>. siRNA therapeutics are already in clinical trials and currently assessed for efficacy and safety. siRNA candidates ARC-520, JNJ-3989 (Arrowhead Pharmaceuticals), and GSK3389404 (Ionis/GlaxoSmithKline)<sup>[122-124]</sup> have displayed promising efficacy in clearing viral transcripts to reduce HBs and HBV DNA production. Small molecules may also achieve the same effect by reducing HBV transcripts. Of note, the small molecule RG7834 was found to be even more potent than using the current clinical therapeutic agent entecavir in reducing HBs levels in cell culture model and HBV-infected human liver chimeric uPA-SCID mice<sup>[125,126]</sup>. With increased interest in natural compounds and their potential antiviral functions, curcumin was shown to reduce the expression of HBe and HBs<sup>[127]</sup> which antagonizes the IFN signalling cascade. Another natural

compound, Dicoumarol, can enhance HBx degradation. It inhibits NQO1 to destabilize its interaction with HBx, rendering it susceptible to the action of proteasomes<sup>[128]</sup>. With such promising new candidate drugs for tackling HBV, combination therapy that couples IFN treatment with these HBV protein-targeting drugs have been proposed to be hopeful for achieving cccDNA elimination without giving HBV the chance to evade the IFN response.

### **Clinical updates on the use of IFNs**

Neither NAs nor IFNs alone can achieve effective HBV elimination in majority of HBV carriers. However, their mechanisms of action complement one another to potentially achieve viral elimination. NAs are effective in suppressing viral titre in most patients, allowing IFNs to effectively mount a cellular immune response in a less immunotolerant environment when HBV titre is decreased, and concurrently allow IFN-mediated intracellular antiviral mechanisms to effectively act against HBV and its cccDNA with less antagonistic effects from decreased HBV titre. As such, combination therapy is increasingly explored, with many experimenting the types of combinations that can be administered (Table 3). Combinations explored include NA monotherapy followed by IFN monotherapy or vice versa, periods of monotherapy followed by periods of IFN and NA co-administration, or co-therapy followed by monotherapy. Interestingly, the efficacies of combination therapy differ greatly. When NA monotherapy is switched to IFN monotherapy, higher rates of HBe and HBs seroconversion are observed together with lower relapse rates. Simultaneous administration of NAs and IFNs for more than 24 wk, followed by sustained NA treatment gives very high HBe seroconversion rate of 50%, accompanied by the loss of HBs expression in 16% patients. This is a remarkable feat considering the loss of HBs is usually less than 5% with NA or IFN monotherapy (Table 1). In contrast, multiple reports show that simultaneous administration of NAs and IFN yield conflicting results, with many studies showing little benefit from adding IFNs into the regime of NA treatment<sup>[129,130]</sup>. Further large-scale clinical studies are needed to ascertain the differences in these findings.

## **CONCLUSION**

It is widely accepted that elimination or silencing of cccDNA is necessary to cure HBV infections so that no active template remains for HBV to reactivate. It is possible for IFNs to achieve this through induction of ISGs, primarily APOBECs that specifically target cccDNA for degradation. IFN signalling also results in the recruitment of epigenetic modifiers that render cccDNA in a transcriptionally repressed and silent state. However, HBV prevents these by inhibiting IFN signalling through upregulating negative regulators of IFN signal transduction such as SOCS2, and sequestration of factors that transduce IFN signalling. Thus, to achieve an anti-HBV response that targets cccDNA specifically, drugs that enhance and sustain the anti-cccDNA effects of IFN signalling and drugs that target HBV products that disrupt these IFN-mediated mechanisms need to be developed. Fortunately, many of the anti-cccDNA mechanisms are not unique to HBV infection. Drugs known to modulate these IFN-mediated pathways can be re-purposed and tested for efficacy in suppressing HBV replication and cccDNA levels. These include LT $\beta$ R agonists and other cytokines that serve as alternative pathways to strengthen IFN-mediated APOBEC expression, inhibitors in the treatment of type II diabetes that act on SOCS proteins to prevent dampening of IFN signalling transduction, and epidrugs that render cccDNA transcriptionally silent. These drugs may be used together with current HBV therapies, new anti-HBV drugs in the pipelines such as cell-penetrating antibodies, cccDNA-targeting CRISPR/Cas9 systems and even natural compounds that directly target HBV proteins that disrupt IFN signalling for degradation. Hopefully, the success of these novel strategies would materialize for the eradication of HBV.

Table 3 Updates on combination therapy

Ref.	Treatment		% loss of HBV markers				% seroconvert		% normal ALT	% HBV reactivation
	Type	Schedule	HBs	HBe	rcDNA	cccDNA	α-HBs	α-HBe		
Hagiwara <i>et al</i> <sup>[160]</sup>	Sim	Combi: 48 wk	4	60	62	85	-	60	77	38
Zhang <i>et al</i> <sup>[63]</sup>	Sim	Combi: 48 wk	9	-	58	-	-	30	73	-
	Mono	IFN: 48 wk	6	-	31	-	-	25	56	-
	Add	IFN: 12 wk; +Combi: 36 wk; +NA: 12 wk	16	-	72	-	-	50	78	-
	Add	IFN: 24 wk; +Combi: 24 wk; +NA: 24 wk	9	-	69	-	-	31	78	-
Huang <i>et al</i> <sup>[48]</sup>	Swi	NA: ≥ 2 yr +IFN: 60 wk	33	91	n.a.	-	26	65	n.a.	-
	Mono	NA: ≥ 2 yr	0	38	n.a.	-	0	22	n.a.	-
Zhou <i>et al</i> <sup>[49]</sup>	Swi	NA: ≥ 4 yr +IFN: 48 wk	36	n.a.	n.a.	-	27	n.a.	-	25
	Mono	NA: ≥ 5 yr	4	n.a.	n.a.	-	0	n.a.	-	58

HBV: Hepatitis B virus; rcDNA: Relaxed circular DNA; cccDNA: Covalently closed circular DNA; ALT: Alanine aminotransferase; NA: Nucleoside/nucleotide analogue; IFN: Interferon; n.a.: Not applicable; Sim: Simultaneous therapy; Add: Add-on therapy; Mono: Monotherapy; Swi: Switch therapy.

## ACKNOWLEDGEMENTS

We thank Zhuo Ziyi for her assistance and comments on the figures.

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