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► **To cite this version:**

David Durantel. Therapies against chronic hepatitis B infections: The times they are a-changin', but the changing is slow!. *Antiviral Research*, 2023, 210, pp.105515. 10.1016/j.antiviral.2022.105515 . hal-04120908

HAL Id: hal-04120908

<https://hal.science/hal-04120908v1>

Submitted on 7 Jun 2023

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Therapies against chronic hepatitis B infections: The times they are a-changin', but the changing is slow!

David Durantel*

Centre International de Recherche en Infectiologie (CIRI), INSERM U1111, UMR_5308 CNRS-Université de Lyon (UCBL1), ENS de Lyon, Lyon, 69007, France

ARTICLE INFO

Keywords:

Hepatitis B virus
Direct acting antivirals (DAAs)
Host-targeting antivirals (HTAs)
Combination therapy
Viro-suppression
HBsAg seroclearance

ABSTRACT

Preamble nota bene: As a tribute to Dr Mike Bray, the following review of literature will be mainly based on published data and concepts, but will also contain my personal views, and in this respect could be more considered as a bioassay.

Even though a cost-effective and excellent prophylactic vaccine exists since many years to protect against hepatitis B virus (HBV) infection, academic-researcher/drug-developers/stakeholders are still busy with the R&D of novel therapies that could eventually have an impact on its worldwide incidence. The Taiwanese experience have univocally demonstrated the effectiveness of constrained national HBV prophylactic vaccination programs to prevent the most dramatic HBV-induced end-stage liver disease, which is hepatocellular carcinoma; but yet the number of individuals chronically infected with the virus, for whom the existing prophylactic vaccine is no longer useful, remains high, with around 300 million individuals around the globe.

In this review/bioassay, recent findings and novel concepts on prospective therapies against HBV infections will be discussed; yet it does not have the pretention to be exhaustive, as "pure immunotherapeutic concepts" will be mainly let aside (or referred to other reviews) due to a lack of expertise of this writer, but also due to the lack of, or incremental, positive results in clinical trials as-off today with these approaches.

List of abbreviations (by order of apparition)

| | |
|-------------------|--------------------------------|
| HBV | hepatitis B virus |
| R&D | research and development |
| HCC | hepatocellular carcinoma |
| WHO | world health organization |
| CHB | chronic hepatitis B |
| NAs | nucleos(t)ide analogues |
| peg-IFN- α | pegylated-interferon-alpha |
| ALT | alanine aminotransferase |
| HIV | human immunodeficiency virus |
| HBsAg | small envelope protein antigen |
| cccDNA | covalently-closed-circular DNA |
| HCV | hepatitis C virus |
| ETV | entecavir |
| TDF | tenofovir disoproxil fumarate |
| TAF | tenofovir alafenamide fumarate |
| pgRNA | pre genomic RNA |

| | |
|---------------|--|
| rcDNA | relaxed-circular DNA |
| VBR | Vebicorvir |
| HBc or Cp | core protein |
| CpAM | core protein allosteric modulator |
| HBcrAg | core-related HBV antigen |
| MoA | mode of action |
| DAA | direct acting antiviral |
| HTA | host-targeting antiviral |
| siRNA or RNAi | small interfering RNA |
| RNaseH | ribonuclease H |
| PAMP | pathogen associated molecular patterns |
| SVP | subviral particles |
| ASO | antisense oligonucleotides |
| TLR | tool-like receptor |
| HBIG | HBV immunoglobulin |
| ADCC | antibody-dependent cell cytotoxicity |
| ADCP | antibody-dependent cell phagocytosis |
| MAb | monoclonal antibody |

* Centre International de Recherche en Infectiologie (CIRI), INSERM U1111, 21 avenue Tony Garnier, 69007, Lyon, France.

E-mail address: david.durantel@inserm.fr.

<https://doi.org/10.1016/j.antiviral.2022.105515>

Received 21 December 2022; Accepted 22 December 2022

0166-3542/© 20XX

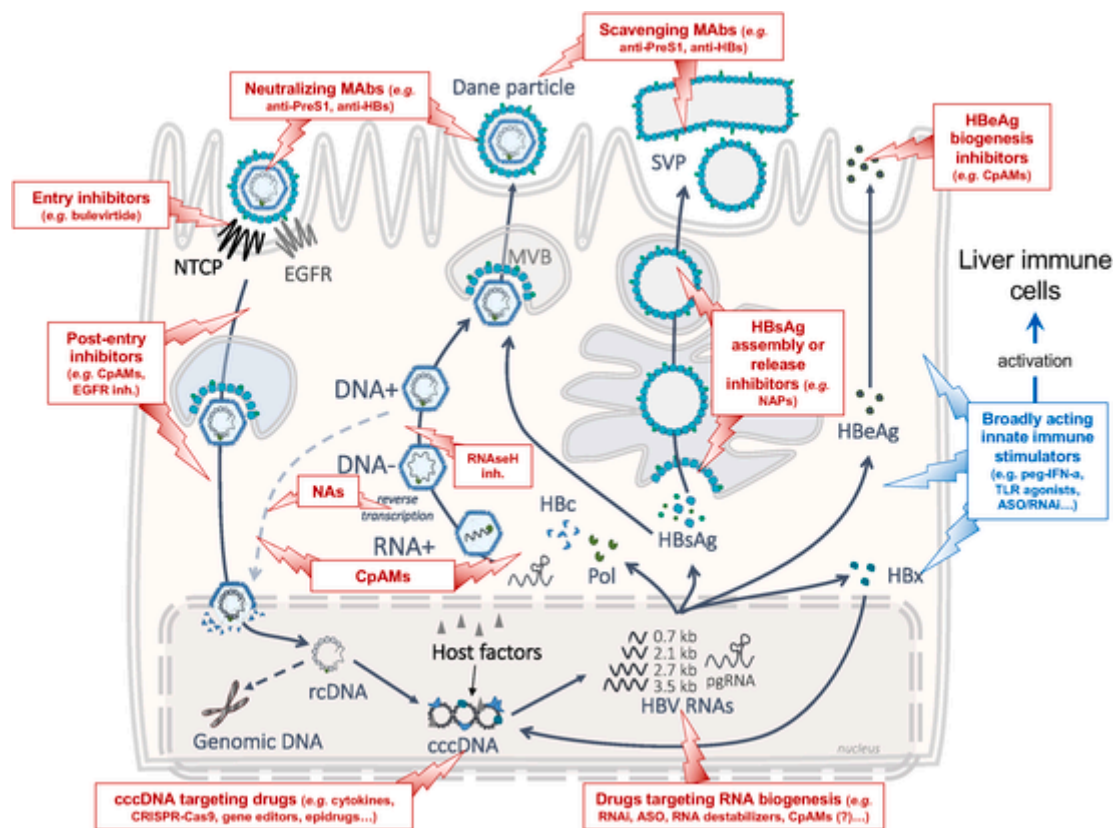


Fig. 1. HBV life cycle and main targets for antivirals based on information reviewed in (Seeger et al., 2015; Tsukuda and Watashi, 2020). HBV enters hepatocytes via the NCTCP receptor; EGF receptor modulates this entry process. The incoming nucleocapsid is released into the cytoplasm and reach the nucleus pore where the HBV genome (rcDNA) is uncoated and delivered to the nucleus. Within the nucleus rcDNA is converted into cccDNA and the latter is “chromatinized” (host and viral factors bind/associate) to become transcriptionally active. From cccDNA, all HBV RNAs can be transcribed including mRNAs and pgRNA. These RNAs are used to encode all viral proteins. PgrNA, covalently associated with one copy of HBV polymerase, is incorporated into the capsid to form immature nucleocapsids. The HBV polymerase performs a reverse transcription of the pgRNA into novel rcDNA within this capsid; a mature nucleocapsid is formed. This mature nucleocapsid has two fates: it can be redirected to the nucleus (“recycling” process) to maintain or amplify the cccDNA pool or it can be enveloped, likely within multi-vesicular bodies (MVB), to generate infectious particles (called Dane particles). Besides these virions two other viral proteins are secreted in large excess: HBeAg and HBsAg, the latter forming subviral particles (SVPs). Most classes of direct and indirect antivirals are indicated on this life cycle in order to pinpoint where there are acting. Abbreviations are listed elsewhere in the manuscript. The picture of the life cycle was edited from reference (Tsukuda and Watashi, 2020), a review already published in *Antiviral Research*.

| | |
|------|------------------------------------|
| PBMC | peripheral blood mononuclear cells |
| FXR | farnesoid X receptor |

1. Current context

Between 250 and 350 million individuals are estimated being chronically infected by the hepatitis B virus (HBV), and at risk of developing end-stage liver diseases (e.g. decompensated cirrhosis, hepatocellular carcinoma (HCC)) (WHO fact sheets). Worldwide, only around 5–10% of chronically infected patients (CHB) are diagnosed, and 1–2% are or have been treated with either nucleos(t)ides analogues (NAs) and/or pegylated-interferon-alpha (peg-IFN- α) (EASL 2017 Clinical Practice Guidelines, 2017; Wong et al., 2022; Yardeni et al., 2023). Treatments are mainly prescribed by *Hepatologists* and only considered when an “active hepatitis” is evidenced notably by elevated seric liver enzyme levels (e.g. ALT) and a risk of progression of fibrosis (EASL 2017 Clinical Practice Guidelines, 2017); this means that there are many identified infected patients that are not treated, including those with a “highly-viremic” HBe-positive infection with normal ALT who can transmit infection by blood contact or sexual intercourse. This rather “atypical” recommendation, compared to other ones in the infectious disease field

(e.g. HIV; for which a treatment is nowadays proposed as soon as the diagnosis is done, whereas back 10 years only the CD4 count prompted decision to treat), has some implications that will be discussed in the last section of this review.

As off today, there are two types of drugs/biologics clinically approved to treat chronic hepatitis B (CHB) patients (EASL 2017 Clinical Practice Guidelines, 2017; Wong et al., 2022; Yardeni et al., 2023). PegIFN- α , a *biologic*, which is weekly and subcutaneously administered for generally 48 weeks, leads to good virological (and biochemical) responses during treatment, but only a weak sustained response (SR) after cessation of treatment, with less than 10% of HBsAg loss/seroclearance; HBsAg seroclearance (6 month after cessation of treatment), associated with virosuppression and associated or not with seroconversion to anti-HBs antibody, defines what is commonly called a *functional cure*. Moreover, peg-IFN- α is often associated with side effects and is poorly tolerated, at least partially due to a systemic exposure to this cytokine (EASL 2017 Clinical Practice Guidelines, 2017; Wong et al., 2022; Yardeni et al., 2023). NAs, which are orally administrated, leads to a strong viral suppression (i.e. reduction of seric viremia) in the majority of patients; the problem of resistance to this class of drugs has been mitigated by the discovery of 2nd generation NAs with high antiviral potency and strong genetic barrier to resistance (i.e. tenofovir or entecavir) (EASL 2017 Clinical Practice Guidelines, 2017; Wong et al., 2022; Yardeni et al., 2023). If the safety profile of NAs is very good to excellent, enabling

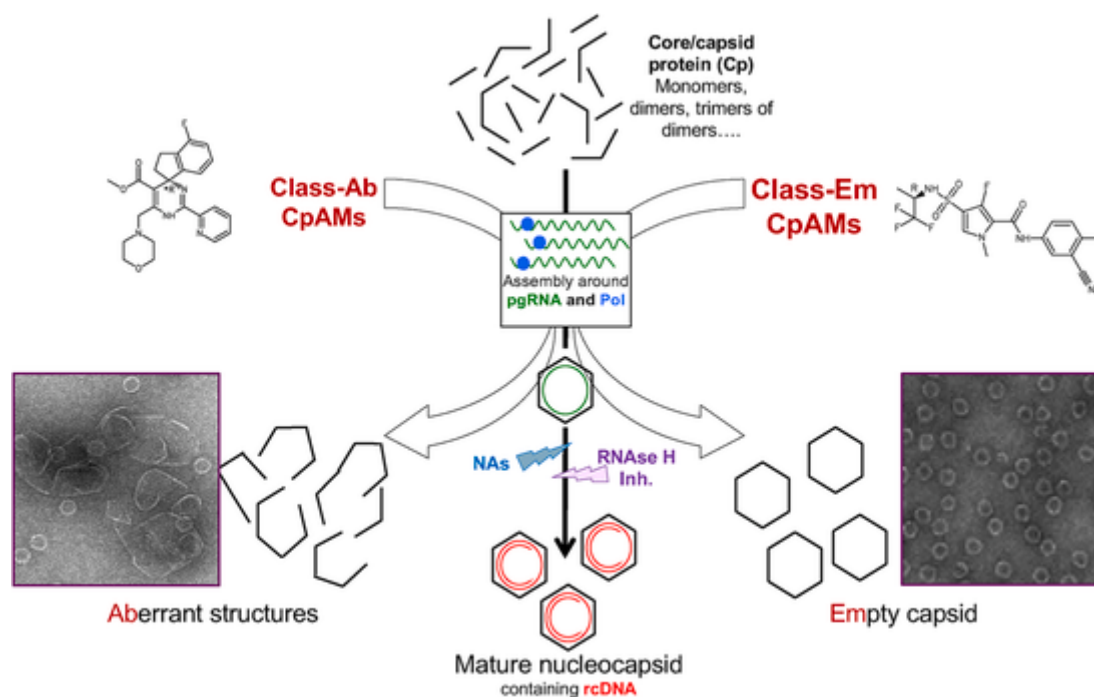


Fig. 2. Molecular mode of action of the two types of CpAMs. There are two classes of CpAMs, which induce the formation of either aberrant or empty capsids. In any cases CpAMs prevent the formation of rcDNA containing nucleocapsids. A similar inhibitory phenotype can be obtained with NAs and/or RNase H inhibitors; this CpAMs, NAs, and RNaseH inhibitors all lead to a decrease in the production of neo-virions, *i.e.* to virosuppression. The two chemotypes of CpAMs indicated, as examples, are the one extracted from the reference (Lahlali et al., 2018) (*i.e.* JNJ-827 and JNJ890).

life-long therapy to prevent relapse of viremia and reduction of the risk of developing HCC, the effect of NAs on HBsAg and cccDNA levels is only marginal (EASL 2017 Clinical Practice Guidelines, 2017; Wong et al., 2022; Yardeni et al., 2023). Yet NAs, when compliance is good, have demonstrated long-term benefits in treated patients by lowering their risks of developing end-stage liver diseases (cirrhosis and HCC) to 2–10 folds healthy population risks (Papatheodoridis et al., 2020).

In order to improve current therapies and move towards more universal and finite duration regimens, faster and stronger, direct or indirect, effects on cccDNA and/or HBsAg levels have to be achieved with novel drugs, together with older ones, and combination of them. The main objective is to increase the rate of *functional cure* (*i.e.* virosuppression + HBsAg seroclearance/loss off-treatment) from 10% to as much as possible (25–30% would be a good step forward (Cornberg et al., 2020)) with novel combination therapies of 2–4 drugs, given either concomitantly or in a sequential manner. HBsAg seroclearance in CHB patients, which can be either obtained naturally or following a treatment, is associated to a greater reduction of the risk of HCC incidence as compared to that obtained with long-term NA-treatment and of course as compared to general population (Wong et al., 2022; Diao et al., 2022a).

As nicely exemplified in the case of HIV, the implementation of more efficient, cost-effective and safe combination therapies is not an easy task (Gibas et al., 2022). Moreover, the “starting” point in the case of CHB is high, with NAs-treated CHB patients who are rather satisfied with their well tolerated “one pill-a-day” treatment and industrial partners who find it more and more difficult to balance the “effort to profit ratio” in this very difficult field of research, despite a great potential market for treating the vast majority of undiagnosed/untreated infected individuals. The last registration of a novel class of drug in the HBV field has been done around 25 years ago; this emphasizes well the difficulty to improve HBV therapeutic strategies and the risk for potential major failures in clinical trials evaluations.

2. Strategies to improve the inhibition of mature HBV nucleocapsid biogenesis, leading to virosuppression

Nucleos(t)ide analogue drugs are potent, safe, and rather unexpensive drugs that are backbones of HIV and HCV therapies, as well as currently used for the treatment of HBV infections (Analogues, 2012; Li and Yue, 2021). Entecavir (ETV), tenofovir disoproxil fumarate (TDF) and its novel prodrug tenofovir alafenamide (TAF), are the three most used 2nd/3rd generation NAs in the HBV field (Pierra Rouviere et al., 2020). In the hepatocyte HBV life cycle, NAs inhibit the reverse transcription of pregenomic RNA (pgRNA) into relaxed-circular DNA (rcDNA); the latter enters into the composition of “mature nucleocapsid” that are subsequently incorporated (by budding into HBsAg-containing host lipid membranes) into neo-produced/secreted HBV virion (Fig. 1). This is the reason why NAs lead to a decline/elimination of viremia in serum, called virosuppression. Moreover, one specificity of the HBV life cycle is that mature nucleocapsids, which contain neo-reverse-transcribed rcDNA, can also be “recycled” to the nucleus of infected cells in order to maintain/replenish cccDNA pool (Fig. 1) (Seeger et al., 2015; Tsukuda and Watashi, 2020). NAs could then theoretically induce, if active at 100% on their enzymatic target, a decline and even a loss of cccDNA in the long term, and this loss of cccDNA could in turn contribute to HBsAg seroclearance. However, one should nevertheless remind that HBsAg can also be made-up from integrated form of HBV and not cccDNA anymore (in particular in HBe-negative patients with low HBsAg levels) (Salpini et al., 2022); in these patients a decline of cccDNA level could have therefore no effect on HBsAg one. But this is only mathematical reasoning with a 100% target engagement. The reality is that, with the second generation of NAs, such an HBsAg clearance is obtained after 10 years of treatment in only few % of cases (0–5% according to studies) in clinic (EASL 2017 Clinical Practice Guidelines, 2017; Wong et al., 2022; Yardeni et al., 2023), thus suggesting that a strict 100% inhibition of HBV polymerase is currently unlikely achieved and that leakiness level of mature nucleocapsids are yet made under treatment and sufficient to maintain cccDNA pool.

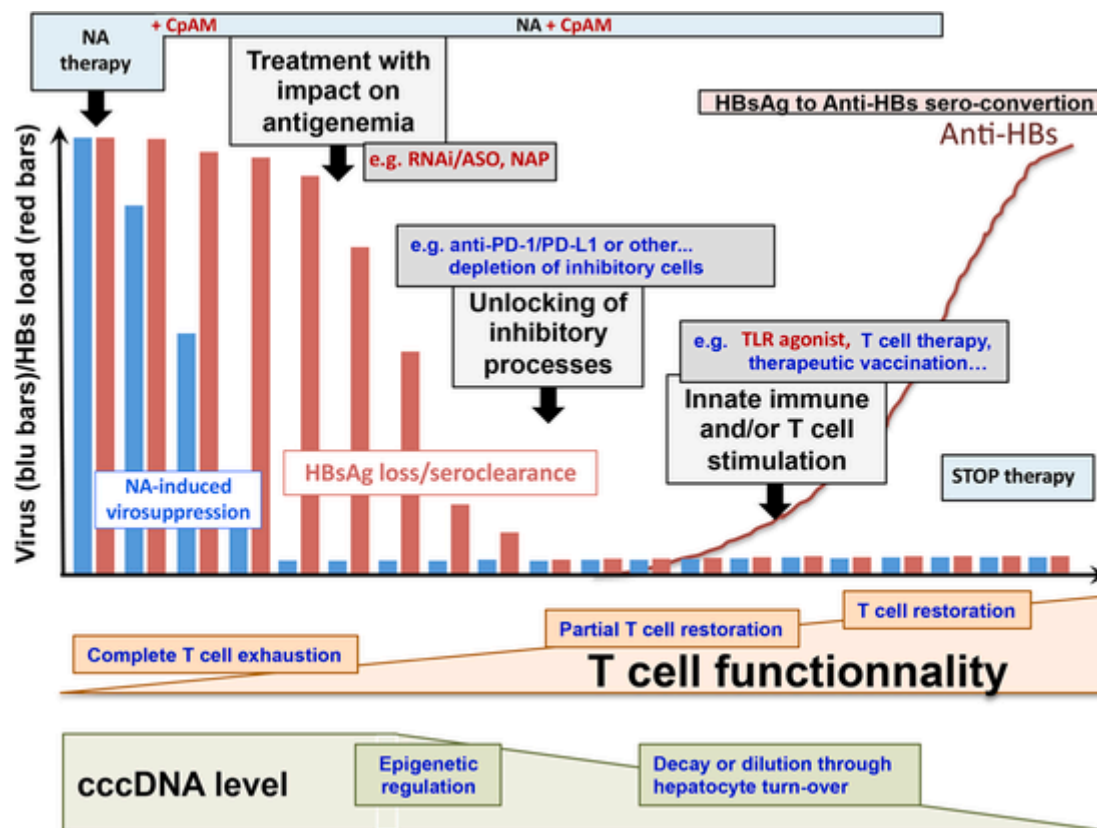


Fig. 3. An illustrated “road map” for the R&D of novel antiviral strategies.

Then, *should more potent 3rd/4th generation NAs be developed?* The relative lack of potency of anti-HBV NAs has been recently evidenced in clinical trials, including for instance the Vebicorvir (VBR) phase-2 clinical trial (NCT03577171), in which VBR, a capsid assembly modulator (CpAM; see after for this class of drug), was combined to ETV. Indeed, it was shown that the combination of ETV + VBR induced a greater viro-suppression, compared to ETV alone, indirectly showing that ETV does not reach full potency at inhibiting mature nucleocapsid formation (Sulkowski et al., 2022). Therefore, *YES, in my view, novel NAs should be developed.* The best anti-HBV NA used in clinic is in the double-digit nanomolar range in term of activity. In the case of HCV, direct acting antivirals (DAAs) active in the picomolar range have been developed and are currently used in clinic (EASL recommendations on treatment of, 2020); some room for improvement does exist for anti-HBV NAs. In this respect, a novel derivative of entecavir (called E-CFCP), which is more potent (0.7–1.8 nM depending on assays), more effective on NA-resistant strains, and has a long-lasting effect *in vitro* and in preclinical mouse animal models, has been described (Higashi-Kuwata et al., 2021) and should be further evaluated in clinical trial. In addition to potency, a better delivery or activation of the NAs within the liver/hepatocytes should also be considered to improve anti-HBV NAs; this concept was recently discussed in another review published in *Antiviral Research* (Pierra Rouviere et al., 2020). Relative to this concept, it is worth noting that ATI-2173, a pro-drug of clevudine and first “Active Site Polymerase Inhibitor Nucleotide (ASPIN)” drug candidate (developed by Antios Therapeutics) has shown improved delivery/activation to liver and interesting phase-1b/2a results (Squires et al., 2022; Tomas et al., 2022). In particular, a longer antiviral activity was observed off-treatment when ATI-2173 was combined to TDF (Squires et al., 2022; Tomas et al., 2022); this could argue for a possible stronger effect on cccDNA, as observed in the woodchuck model with clevudine derivatives (Hui and Lau, 2005; Peek et al., 2001).

Another way to improve inhibition of the mature nucleocapsid formation and subsequent viro-suppression could come from the R&D of drugs targeting HBV nucleocapsid assembly; these drugs are mainly called “core assembly modulators” (CpAMs) (Viswanathan et al., 2020). CpAMs act “upstream” of NAs (in the pathway of mature nucleocapsid formation) by preventing the incorporation of pgRNA into capsid and therefore its subsequent conversion into rcDNA by HBV polymerase; the latter being exclusively active within an already formed capsid (Fig. 2) (Seeger et al., 2015; Tsukuda and Watashi, 2020). Many CpAMs, belonging to two main classes in terms of mode of action (Class-Em or Class-Ab) have been identified and some of them evaluated in clinical trials (Yardeni et al., 2023; Viswanathan et al., 2020). The first and second generation of CpAMs (e.g. BAY41-4109, AT130, NVR 3-778, JNJ-6379/Bersacapavir, Vebicorvir/ABI-H0731 ...) were not potent enough (and/or not safe enough) in monotherapy or combination therapies in clinical trials and have been either discontinued or slow-down/put in hold in their clinical development. What was expected with CpAMs alone, or in combination with other molecules, was a faster decline of HBsAg levels, which could theoretically be explained by an improved inhibition of mature nucleocapsid formation leading to a faster cccDNA decline following a “full stop” of the “recycling” phenomenon. Maybe expectations were initially too high regarding these CpAMs, whose main mode of action is not a direct inhibition of HBsAg expression/secretion and the conditions of their evaluation too harsh, with expected results in the short term. First and 2nd generations of CpAMs were however rather good at reinforcing viro-suppression in combination with NAs, as demonstrated in Vebicorvir trials (Sulkowski et al., 2022; Yuen et al., 2022a), and this could already be interesting enough whether treatments could be extended to several years; yet the current evaluation “standard”, for novel therapy design and demonstration of efficacy in clinical trials, is a 48-week treatment period for a short finite duration regimen aiming at an increase of HBsAg seroclearance rate to around 30%. Maybe this standard is too ambitious and should be revis-

ited to give another chance, in particular to CpAMs that are overall great DAAs. Nevertheless, the lack of potency of previous generations of CpAMs has been now tackled and more potent (ultra-potent) CpAMs (with EC₅₀ going from hundreds of nM down to single digit nM or even pM range (Unchwaniwala et al., 2022; Zhang et al., 2020); ABI-4334, ATI-1428, AB-836, ALG-000184 ...) have now entered phase-1 clinical trials (Gane et al., 2022a). Hopefully these novel CpAMs will be evaluated with better clinical endpoints and in longer clinical trials to obtain a full power of this class of drugs.

In contrast to NAs, CpAMs are also capable to reduce seric HBV RNAs and also to some extent secreted HBcrAg (core-related HBV antigen; *i.e.* a composite marker made of secreted forms of Hbc and HBe proteins) (Sulkowski et al., 2022; Yuen et al., 2019, 2020a, 2021a, 2022a; Zoulim et al., 2020). Moreover, *in vitro*, CpAMs were shown to have several modes of action beside their primary target, which is of course the assembly of nucleocapsids. Indeed, at a higher concentration, they have been shown to block cccDNA establishment (secondary MoA) (Berke et al., 2017; Lahlali et al., 2018), HBeAg biogenesis (tertiary MoA) (Lahlali et al., 2018), as well as HBV RNA biogenesis for the most potent of them (quaternary MoA) (Fig. 1); the latter was evidenced *in vitro* and in animal models in very long-term treatment conditions (Lahlali et al., 2018) (and *our unpublished data*). This inhibitory effect on HBV biogenesis is likely due to the regulatory functions of the Hbc/core protein, which can be found associated with cccDNA and HBV RNAs in the nucleus of infected hepatocytes (Lucifora et al., 2021) (and *our unpublished data*) and likely plays roles in cccDNA transcription and post-transcriptional events (Chabrolles et al., 2020; Taverniti and Ligat). All these additional MoA are dependent on the concentration of CpAMs and therefore on their potency. It is therefore expected that 4th generation CpAMs, which are ultra-potent could lead to additional inhibitory phenotype *in vivo*, including a faster and stronger reduction of cccDNA and consequently HBSAg levels. Repetitive failures of CpAMs (of 1st/2nd generations) to demonstrate a real “added value” in combination with NAs and/or RNAi in various clinical trials have almost disqualified this class of drugs; but great hopes reside in the 3rd/4th generations of CpAMs.

Is there a place for HBV ribonuclease H (RNaseH) to improve viro-suppression? NAs target the reverse transcription activity of the HBV polymerase. But HBV-pol contains also a RNaseH domain which is important to degrade RNA templates during the RT process (Edwards et al., 2019); this enzymatic activity is considered as a possible drug target, with the identification of three different chemical families that inhibit its activity (Edwards et al., 2019; Li et al., 2021a); Structure-activity-relationship studies are on-going to improve the potency and toxicological profile of these drugs, which are rather low as off today. Mechanistically speaking, these inhibitors should reinforce the inhibition of mature nucleocapsid formation, which can be obtained with NAs and/or CpAMs (Fig. 1). In addition, blocking the RT process in-between pgRNA and rcDNA could generate non-physiologic replication intermediates (RNA/DNA hybrids), which in turn may serve as PAMP (pathogen-associated molecular patterns) to activate innate immunity/IFN response. Knowing that HBV infected hepatocytes are immunologically “cold” (because HBV is a rather stealth virus (Wieland et al., 2004)), a strategy that could turn these “cold” cells into “hot” ones (*i.e.* cells producing alarming IFNs or cytokines) is worth investigating. Combination of CpAMs and RNaseH inhibitors, which could reinforce this phenotype, could be interesting to test in that respect.

3. Strategies to, directly or indirectly, improve HBSAg loss/seroclearance

All strategies that will have an impact on the level or transcriptional activity of cccDNA will end up having an impact on HBSAg level, as cccDNA is the main template for all HBV RNA biogenesis (Seeger et al., 2015; Tsukuda and Watashi, 2020); the exception to this is found in

HBeAg negative patients well advanced in their natural history, in who HBSAg can also be significantly expressed by integrated HBV into host genome (Salpini et al., 2022). A section on the direct targeting of cccDNA level and activity will be found just after. Building up on the previous section and concepts, in theory, a complete level of viro-suppression and 100% inhibition of the formation of mature nucleocapsid, should be good enough to allow cccDNA decline by both blockade of the reinfection of novel hepatocyte and blockade of the recycling of mature nucleocapsid, subsequently leading to HBSAg decline. So far, we have discussed about NAs, CpAMs and RNaseH inhibitors. In this section we will focus on other antiviral molecules that can have a more direct impact on HBSAg biogenesis and/or secretion and/or seric level. Some of these molecules could also reinforce the inhibition of mature nucleocapsid biogenesis and therefore viro-suppression, thus showing that feed forward amplification loops are at play.

Small interfering RNAs (siRNA or RNAi) targeting HBV sequences can induce a rather specific degradation of all HBV RNAs, provided that they are designed to target a relevant 3' sequence common to all RNAs. By doing so HBV-targeting RNAi are theoretically capable to suppress the expression of all HBV proteins (HBx, HBSAg, Cp, etc ...) and synthesis of pgRNA, which are all instrumental for the generation of viral progeny (van den Berg et al., 2020) (Fig. 1). As HBV proteins are endowed with crucial proviral functions (HBx allows cccDNA transcription by inducing degradation of the restriction factor Smc5/6 (Decorsiere et al., 2016); Cp allows capsid formation (Seeger et al., 2015); HBV-pol allows reversion transcription (Seeger et al., 2015; Tsukuda and Watashi, 2020); envelop proteins allow virion and subviral particles (SVPs) formation (Seeger et al., 2015; Tsukuda and Watashi, 2020), ...), a feed forward amplification loop of inhibitory phenotypes is expected, and HBV-RNAi could be seen as an “holy grail antiviral” (van den Berg et al., 2020). But the main problem of this class of biologic/antiviral resides in its delivery to the liver and within the liver to all infected cells. This galenic problem is far from being futile and likely the reason why so far clinical trials have led to good, but not outstanding, results, in particular in monotherapies. So far clinical-stage RNAi have been either embedded in proprietary lipid formulations or conjugated with moieties known to allow hepatocytes delivery (*i.e.* essentially N-acetylgalactosamine; GalNac) (van den Berg et al., 2020; Dowdy and Levy, 2018; Hui et al., 2022; Springer and Dowdy, 2018). There are currently 5 RNAi (JNJ-3989, VIR-2218, AB-729, ALG-125755, RG6346), evaluated in monotherapies and/or combination in clinical trials (Fitzgerald et al., 2022; J. Hepatol. 73, S20, 2021a, 2021b; Lim et al., 2022; Yuen et al., 2020b, 2021b, 2022b, 2022c). They mostly target the HBx region within genome, leading to virtual degradation of all HBV RNAs (due to 3'overlapping of all HBV RNAs), including RNAs coming from integrated HBV genomes; but recently RNAi targeting envelope protein genes have been R&Ded. These RNAi gene silencers are generally rather safe in monotherapies and associated to rather potent and durable HBSAg declines (1–3 log₁₀; sometime higher leading to HBSAg seroclearance in few patients). Interestingly there are subcutaneously injected in a monthly (or even less frequently) manner, which is very convenient for patients. The absence of ALT flares when used in monotherapies, which is a good thing when safety is concerned, could nevertheless be seen as a problem, as moderate and clinically controlled ALT flares (so called “good flares”) might be needed to foster HBV cure. This also suggest that HBSAg decline may not be sufficient to restore immune responses against infected cells, as initially thought. Combination of RNAi with immune stimulator might be needed to improve the rate of *functional cure*; this will be discussed in the combination section.

Antisense oligonucleotides (ASO) can also be used to block the translation of HBV mRNAs, including HBSAg ones. One ASO has reached phase-2 clinical development; it is GSK3228836/Bepirovirsen (Yuen et al., 2021c), which is a “naked” antisense oligonucleotide (a version with GalNac moiety, *i.e.* GSK3389404 was less efficient (Yuen

et al., 2022d)). This asset led to a profound decline of HBsAg level, 3–4 log₁₀, after only 28 days of administration (once a week subcutaneously). As opposed to RNAi, moderate elevations of ALT could explain potency and durability of the HBsAg decline/seroclearance. In the very recently published B-Clear phase-2b study (Yuen et al., 2022e), it was shown that a dose of 300 mg per week for 24 weeks led to a sustained response in terms of HBsAg decline below lower level of detection (*i.e.* *functional cure*) in 9–10% of patients with CHB, who were either co-administrated or not with a NA. Even if not reaching undetectability, 65–68% of patients treated with Bepirovirsen (alone of with a NA), had their HBsAg level going below 100 IU/mL, which is very encouraging for this drug. Longer treatment periods are currently investigated to determine whether the rate of *functional cure* could be improved upon; even if such an improvement is not observed, the durability of decrease of HBsAg under 100 IU/mL could allow stopping Bepirovirsen and the associated NA, as well as investigating *functional cure* post-treatment (see concept of stopping NA strategy here after). The safety of this drug is yet closely investigated (B-Sure trial; NCT04954859), as few patients had to stop the treatment during trials because of drug-induced adverse effects. The possibility that Bepirovirsen is more active than RNAi because of a possible activation of TLR sensors (likely TLR8; but TLR9 was also evoked) is being investigated (You et al., 2022); such a possible dual mode of action (*i.e.* inhibition of mRNA translation + innate immune stimulation) is for sure in the spirit of what need to be done to *functionally cure* HBV (see later). More results from trials with Bepirovirsen alone or in combination with other assets are expected soon.

Acting downstream of RNAi and ASO in the HBV life cycle, nucleic acid polymers (NAPs; *e.g.* REP 2139) have also been shown to rapidly reduce circulating HBsAg or subviral particles (SVP) (Vaillant, 2022a, 2022b). Indeed, REP 2139 neither induce HBV RNA degradation, nor inhibit HBsAg mRNAs translation in infected hepatocytes, but it prevent HBsAg secretion by likely targeting the host-factor DNAJB12 involved in subviral particles (SVPs) biogenesis (Vaillant, 2022a). The anti-HBV activity of all NAPs is sequence independent and driven by a length-dependent (40-mers being optimal) and phosphorothioation (hydrophobic)-dependent interaction with amphipathic alpha helices present in target proteins (Vaillant, 2022a, 2022b). In a recently published phase-2 trial study, it was shown that the addition of REP 2139 to TDF + peg-IFN- α significantly increased the rate of HBsAg loss (associated with anti-HBs antibody seroconversion) after 48 weeks of therapy. A *functional cure* was observed in 14 of 40 participants (*i.e.* 35% of cases), which is an unprecedented rate of cure in the CHB field (Bazinet et al., 2020). However, there are several drawbacks for the use of REP 2139: it has to be combined with peg-IFN- α for a full efficacy, it has to be administrated every day by intravenous infusion, and safety profile in the long-term is yet to be investigated in larger cohorts of patients (Durantel and Asselah, 2020).

Two final types of assets able to reduce HBsAg levels are also worth mentioning.

First, small molecules of the dihydroquinolizinone chemical series, including orally available RG7834 (initially developed by Roche), were identified as able to accelerate the degradation of HBsAg mRNAs and therefore to reduce intracellular HBsAg synthesis/expression in infected cells and in turn HBsAg/SVPs secretion (Mueller et al., 2018). RG7834 was shown to target PAPD5/7 host factors, which are involved in the polyadenylation of HBV RNAs and their stabilization (Mueller et al., 2019). The first generation of these drugs was shown to be very toxic (neurotoxic in particular) *in vivo*; this could have been anticipated due to the fact that PAPD5/7 do stabilize also host RNAs. Efforts to improve the delivery of this type of drug to the liver or to increase its specificity of action on HBV RNAs (and not host RNAs anymore) are being made by several academic and corporate researchers to give rise to second generation compounds with better safety profiles (*e.g.* GSK3965193, GST-HG131 ...) (Li et al., 2022).

Finally, another class of molecule is anti-HBs antibody. Immunoglobulins (HBIG; mainly targeting HBsAg) isolated from vaccinated patients have been used since many years for passive prevention of reinfection of liver grafts and mother-to-child transmission (Chen et al., 2020; Orfanidou and Papatheodoridis, 2021). Injection of these immunoglobulins in CHB patients had in contrast little value. But recent research in the fields of HIV and more recently Sars-CoV2 have revived interest on monoclonal antibodies endowed with neutralizing capacity, as well as ADCC (Antibody-Dependent Cell Cytotoxicity), ADCP (Antibody-Dependent Cell Phagocytosis) (and other) properties. Quite recently, broadly monoclonal neutralizing antibodies have been identified in natural controllers of the HBV infection or in vaccinees and characterized (Beretta and Mouquet, 2022; Hehle et al., 2020). Concomitantly several monoclonal antibodies directed against the main antigenic loop (the “a” determinant) in the HBsAg protein (*i.e.* small HBs protein) have been reported (Burm et al., 2023; Golsaz-Shirazi et al., 2017; Kucinskaite-Kodze et al., 2016; Wang et al., 2016; Wu et al., 2022; Zhang et al., 2022). The best MAbs should recognize conformational epitopes, have strong and broad neutralizing activity (EC₅₀ at single digit ng/mL, or below) and ideally should have been engineered in their Fc domain to enhanced ADCC and/or ADCP functionalities; this is the case of VIR-3434 (VIR biotech) and Lenvirmab (GC Pharma) (Wu et al., 2022; Gupta et al., 2021), which have successfully entered phase-2 clinical studies, following the evaluation of their safety and efficacy on HBsAg levels in phase1/2a studies. Their combination with other assets should be interesting.

4. Strategies to target cccDNA

CccDNA represents the ultimate target in the HBV life cycle in order to get a *functional cure* and maybe, in a far future, a more *complete cure* (Martinez et al., 2021; Seeger, 2018). As already mentioned, all strategies that will have an impact on the level (*i.e.* by degradation or loss through cell division) or transcriptional activity (*i.e.* transcriptional repression) of cccDNA should end up having an impact on HBsAg level, as cccDNA is the main template for all HBV RNA biogenesis. And, as some viral proteins (*e.g.* HBx, and maybe Cp) are important to maintain cccDNA transcriptional activity, a feed forward inhibitory amplification loop could be expected.

A degradation and/or an inhibition of the transcriptional activity of cccDNA has been mainly observed *in vitro* with innate immune cytokines, including interferons, lymphotoxin beta receptor agonist, TLR2 and TLR3 agonists, and several pro-inflammatory cytokines (*e.g.* IL-6, IL-1b ...) (Delphin et al., 2021; Durantel and Zoulim, 2016; Isorce et al., 2015, 2016; Lucifora et al., 2014, 2018; Xia et al., 2016). As off today, only IFN- α (mainly in its pegylated form) is used in human; although its MoAs in human have not been completely elucidated, it is likely that part of the antiviral activity of this drug, which can lead to around 5–10% of *functional cure*, is due to a decline in cccDNA transcriptional activity, as well as a direct (non-cytopathic) or indirect (via immune driven cell death \pm hepatocyte division/turn-over) degradation/loss of cccDNA. As IFN- α is less potent than proinflammatory cytokines to induce a cccDNA degradation and/or transcriptional arrest *in vitro*, it would be interesting to develop an asset that could induce the local production of these cytokines (*i.e.* IL-1 β , IL-6, LT β ...). So far two clinically tested types of molecules are capable to induce the production of such cytokines *ex vivo* and *in vivo*: there are TLR7 and TLR8 agonists. Both types of agonists were shown to induce the production of cytokines in isolated PBMC or/and *in vivo*, as well as were also shown to induce a strong decline in cccDNA in the woodchuck model (Amin et al., 2021; Li et al., 2018; Menne et al., 2015; Niu et al., 2018). Yet the TLR7 agonist GS9620/Vesatolimod was shown not potent enough in monotherapy in CHB patients at the doses tested (Gane et al., 2015) and the TLR8 agonist GS9688/Selgantolimod is also facing problem of efficiency in monotherapy in on-going clinical trials (Gane et al., 2022b); the latter is

also investigated in combination with other assets or in other strategies. To summarize, so far IFN- α has not been yet replaced by another immune-stimulator in clinical practice; but such a goal is yet on the table. Beside TLR7 and TLR8 agonists, there are some investigations on TLR2 and TLR3 agonists, which could also be of interest in that respect (Lucifora et al., 2018; Desmares et al., 2022). It is however worth noting that RIGI and NOD2, despite initial interest (Yuen et al., 2022f), are not considered anymore as targets due to a fatality in a phase-2b clinical trial with Inarivir, a RIGI/NOD2 agonist.

In the category of pure epigenetic modifiers, none of the epidrugs that could have shown anti-HBV activity *in vitro* (Dandri, 2020; Zeisel et al., 2021), have reached clinical trial stage, maybe with the exception of farnesoid X receptor (FXR) agonists. Two FXR agonists, EYP001 (Enyo Pharma) (Darteil et al., 2019; Erken et al., 2018) and ASC42 (Ascleris) are in phase-2 trials. Of note these two drugs are also clinically tested against NASH, for which FXR has been identified as a relevant target (Li et al., 2021b; Radreau et al., 2019). *In vitro* these drugs lead to an HBsAg decline following a partial cccDNA silencing (Barnault et al., 2022; Li et al., 2021c). Combination of EYP001 and IFN- α *in vitro* and in an open-labeled phase-2 trial (EYP001-203) has shown interesting results on HBsAg levels, thus suggesting that the combination of two assets able to each target cccDNA transcriptional activity could be a good strategy (Barnault et al., 2022).

Finally, as of today, strategies based on Crisper-Cas9 or gene editing technologies to either degrade or de-functionalized cccDNA are yet at the preclinical stage (Martinez et al., 2022); further development will request gallenic efforts (not to say miracle) to efficiently deliver “active assets” (whatever they are; proteins, expression vector etc ...) to the site of replication of the virus. Moreover small chemical molecules that would be able to induce a specific cccDNA degradation do not exist and are unlikely to be discovered!

One efficient way to loose cccDNA is to induce the division of infected hepatocytes; indeed it is now rather well proven, *in vitro* and in mouse animal models, that cccDNA does not resist to cell division (Lutgehetmann et al., 2010; Tu et al., 2022). Quantifying the number of hepatocyte division in an individual “normally” behaving (*i.e.* taking acetaminophen or other hepatotoxic medics from time to time, consuming alcohol even at low dose, having different food habits, etc ...), which we could define as micro-regenerative events, is a difficult task; yet such hepatocyte turnover could play a role, and explain differences at individual level, in the natural history or treatment history of CHB patients. Another way to loose cccDNA is to kill infected cells via a targeted immune response for instance; this is the goal of some immune therapies (therapeutic vaccination, T-cell therapies, checkpoints inhibitors ...), which would not be discussed in this review in details, but can be viewed in the following recent reviews (Fanning et al., 2019; Gehring and Protzer, 2019; Maini and Burton, 2019). Induced death of infected cells, as a broad therapeutic concept, has to be handled with care as it is not clear how many hepatocytes are infected at a given stage of the natural history of HBV infections, and one want to avoid “bad ALT flares” that could lead to liver failure. Considerations on “bad versus good flares” (the latter being looked for to improve *functional cure* rate) can be found in other reviews (Ghany et al., 2020; Liaw, 2022); with our current understanding, it seems impossible to think that an increased rate of *functional cure* can be obtained in “short duration regimen” without “playing” somehow with good flare events. Looking for finite duration regimens to replace safe but almost long-life administration of NAs, has a cost!

5. Combination strategies

Combination of assets is unanimously seen as the way forward to design a short (or one should say “not too long”) finite duration regimen able to significantly increase the rate of *functional cure* (Wong et al., 2022; Yardeni et al., 2023; Durantel and Zoulim, 2016; Fanning et al.,

2019). Many combinations involving investigational drugs CpAMs, RNAi/ASO, NAs, MAb, etc ..., between them and/or with approved ones (*i.e.* NAs and peg-IFN- α) are on-going in many registered clinical trials (*c.f.* to lists into (Wong et al., 2022; Yardeni et al., 2023)). The aim of combination therapies is to get stronger (hopefully synergistic) or/and faster inhibitory phenotypes on given parameters in order to increase as much as possible the % of *functional cure* defined as sustained virosuppression + HBsAg loss (associated or not with anti-HBs sero-conversion; 6–12 months off-treatment), while achieving at *minima* superior and durable virosuppression and/or HBsAg declines (several logs, even if no proper *functional cure* is achieved) as compared to each asset evaluated separately. And, of course, the safety profile should be as good as each single element of the combination, proving no negative drug-drug interactions; one has to reckon that most of the clinical evaluations failed to date because of lack of superiority in term of efficacy and more importantly because of inferiority in terms of safety/tolerability.

So far with all combination tested in phase-2 to date, only very few succeeded in increasing the % of *functional cure*, which was, before all these recent efforts, best achieved by the combination of peg-IFN- α plus TDF in clinical trial condition (around 8–9%) (Marcellin et al., 2016). Among successful combinations, is the complex combination of NA + peg-IFN- α + REP 2139, which has led to a 30–35% *functional cure* after 48 weeks of treatment (Bazinet et al., 2020). In this trial, ALT elevations were very high, necessitating particular clinical care, but did not lead to liver failure. As discussed previously the use of REP 2139 is tricky because of the route and number of injections needed and further optimization (*e.g.* gallenic of the drug) and clinical evaluations are required (Durantel and Asselah, 2020). Quite disappointingly, the final results of a triple combination of 3 DAAs, *i.e.* NA + CpAM + RNAi (REEF2 study, NCT03365947; 48 weeks of NA alone versus NA + RNAi/JNJ-3989 \pm CpAM/JNJ-6379, followed by 48 weeks of treatment) (*c.f.* Agarwal et al., AASLD, 2022; Abs. LB 5012) did not show any *functional cure*, while allowing an interesting long-lasting drop of HBsAg level under 100 IU/mL in 45% of patients versus 15% (in NA alone arm). Very disappointingly, the additive value of CpAM was only incremental; but 4th generation CpAMs could be a game changing in that respect. And of course, one should admit that longer duration of treatment are needed to further evaluate this concept of combination of direct acting agents (DAAs). To shorten combination therapies of DAAs, this reviewer does think that a “host targeting” immune component will be needed. This could be an innate-immune stimulators (TLR7 or 8 agonist, or peg-IFN- α itself; see next section for this latter reshuffled concept), a checkpoint inhibitor (anti-PD1/PDL1) (Wang et al., 2016; Gane et al., 2019), one (or two; targeting both the main “a” determinant of HBs and the preS1 domain?) monoclonal antibody with enhanced Fc functions or/and a more sophisticated immune therapeutic component (therapeutic vaccination, CAR/TCR T cell adoption, etc ...) (Fanning et al., 2019; Gehring and Protzer, 2019; Maini and Burton, 2019). Great hope resides in Bepirovirsen, which is a possible “double bullet” asset (inhibition of HBV RNA translation/HBV protein expression + possible TLR8/9 agonist) and could be combined to a NA (TDF or ETV) or added-on (for CHB patients already under NA-treatment) to set a sort of triple therapy featuring 2 DAA activities plus one innate immune one. Bepirovirsen was announced it will be evaluated in 2023 in a phase-3 trial (the first one to be launched since NAs were implemented in the HBV field) with several arms; the times they could be a-changin’!

6. Playing with old drugs: “back to the past” pragmatic approaches

Interestingly, but also sadly (when the economy of R&D of novel drug is concerned), the best successes in terms of *functional cure* have been recently obtained with old medicines and concepts.

In particular the “stopping NA” strategy has gained great interest and has been extensively investigated recently in clinical studies and practices (Wong et al., 2022; Yardeni et al., 2023; Sonneveld et al., 2022). This consists of stopping the administration of the NA in patients who have been treated for a (very) long period of time (> 48 weeks), and monitor whether the viremia will either i) rebound and a productive infection restart (necessitating re-treatment), ii) remain low off-treatment (with no necessary change in HBsAg level; in this case no re-treatment needed, only observation), or iii) further get down with an HBsAg seroclearance. This strategy is applied almost exclusively to HBe-negative patients with already, naturally or treatment-induced, rather low HBsAg levels (< 500–3000 IU/mL; depending on local practice and studies). Indeed, it was shown that very low HBsAg level (e.g. < 100 IU/mL), as well as HBV genotype (genotype C would be more favorable), was associated with greater chances of achieving a *functional cure* (Wong et al., 2022; Yardeni et al., 2023; Sonneveld et al., 2022). Stopping NA can be associated with ALT elevation that can be a sign for either “good or bad flares”, which could either shift the balance toward a possible cure (flares will be retrospectively called good flares) or serious liver events (decompensation, liver failure ...) (flares will be retrospectively called bad flares) (Tseng et al., 2023). Predictors (beyond HBsAg level and HBe-negative status) of HBsAg seroclearance are actively looked for, including immunological ones, in order to better identify CHB patients who would benefit the most from this strategy. More information of “stopping NA” strategy can be found in these reviews (Wong et al., 2022; Berg and Lampertico, 2021; Jeng et al., 2021; Papatheodoridi and Papatheodoridis, 2021), whereas the concept of good versus bad flares is discussed in these ones (Ghany et al., 2020; Liaw, 2022). Whether combinations of DAAs (e.g. NA + CpAM + RNAi) would replace current NA-based treatments in near future, then the stopping concept would also apply to these novel therapies; this is why the on-going intensive search for biomarkers/immune-markers that could predict for HBsAg seroclearance after stopping treatment is crucial.

Peg-IFN- α , which was first used (in a non-pegylated form) to treat HBV infections in the eighties, has recently made a strong come back in therapeutic strategies, despite its poor toxicological and tolerability profiles. Historically, interferon from lymphoblastoid origin remains the first medicine that allowed reported HBsAg seroclearances in CHB patients (Alexander et al., 1987). Thirty-five years later, peg-IFN- α is included in treatment arms of many on-going and planned phase-2 trials in double (or triple) combination with CpAMs, RNAi (e.g. VIR-2218), ASO (bepirovirsen), etc... In combination with the NAP REP 2139, and on the top of a NA, Peg-IFN- α led to the greatest rate of *functional cure* ever reported and published to date (Bazinet et al., 2020). Moreover, it has recently been successfully used, off-label, in combination with bulevirtide (an HBV/HDV entry inhibitor) to improve treatment of patients (except those with decompensated cirrhosis) chronically infected with the hepatitis delta virus (Lampertico et al., 2022).

As other innate immune-modulators, such as TLR agonists, struggle to make it to the clinic, Peg-IFN- α remains a therapeutic option. In the last 10–15 years, while efforts to develop novel DAAs and HTAs were intensifying, many clinical studies and reports on clinical practices have kept reminding us that peg-IFN- α continue to be a therapeutic option, in particular in combination. The first convincing 48-week *de novo* combination of peg-IFN- α plus TDF, showing an 8–9% HBsAg seroclearance in a clinical trial setting, was reported in 2016 by Marcellin and colleagues (Marcellin et al., 2016). While many others *de novo* combination studies with NAs have been performed and reported (with more or less patients, in different geographical area with genetically different population, with different HBV genotypes, etc ...), other schedules were also tested: add-on of peg-IFN- α in NA-suppressed patients, switching to peg-IFN- α after NA-suppressing treatment, lead-in period of peg-IFN- α before switching or adding to another drug. A recent meta-analysis of this exhaustive literature has been published and has concluded that in

NA-virosuppressed patients, switching to peg-IFN- α was superior to the add-on strategy, and that both switching and add-on strategies were superior to NA continuation alone regarding the rate of HBsAg seroclearance (Liu et al., 2020). The latest large multicenter real-world study in NA-suppressed HBeAg negative CHB patients (Everest project in China; NCT04035837) reported interim results with as high as 33% of *functional cure* after 48 weeks in *per protocol* analysis (Wu et al., 2021); and authors claim that “(dixit) “There are still good chances of *functional cure* for more than half of the patients who had not achieved HBsAg loss by prolonged treatment or retreatment”. *Are best soups made in old pots?* One should be cautious about this, as the use of peg-IFN- α , whatever the schedule, is frequently associated with ALT flares, which have to be clinically managed in expert *Hepatology* center. We are still far away from a possible, easy, twice-a-year administration as awaited with lenacapavir in the HIV field (Marrazzo, 2022) !

7. Conclusions and final considerations

The objective of this review/bioassay was to, non-exhaustively, discuss about the main novel (or old, but reshuffled) therapeutic strategies, which are currently clinically evaluated, in the field of HBV chronic infections.

But before concluding and further debating on the necessity to carry on efforts to design and implement purely novel strategies to cure HBV infections with a finite regimen, let us play to the devil's advocate. The best recent successes in terms of improved *functional cure* for HBV were obtained by using old drugs: namely NAs (*c.f.* to stopping strategy) and peg-IFN- α (mainly used in combination). Why therefore not scaling-down our efforts to develop new types of drugs & scaling-up our effort to identify undiagnosed patients (*i.e.* 90-95% of infected patients worldwide would not be aware of their status) and put them on treatment (< 1–2% of the 300 million of individuals potentially chronically infected are treated!), as well as enforcing prophylactic vaccination programs? In line with this reasoning, one should keep in mind that there are plenty of patients, whose HBV sero-positivity is known, that are not treated because their liver is doing well! This “atypical” recommendation of not treating patients who could have elevated HBV viremia and be in capacity to transmit infection to others could evolved. In particular, it is now well established that HBV integration into host genome does occur very rapidly after the onset of infection and keep accumulating over time if the replication of the virus is not stopped by NAs for instance, thus increasing the risk that these integrative events may become, 5–30 years after, a component of HCC initiation and progression (Salpini et al., 2022; Kim et al., 2018; Mason et al., 2016; Tu et al., 2018, 2021). Why not treating (with the safe NAs we dispose of) the patients who were previously called “immune tolerant” (now referred as HBe-positive patients with a chronic infection (EASL 2017 Clinical Practice Guidelines, 2017)) as soon as there are diagnosed (Bertoletti et al., 2018)? This would make sense, not only with respect to early integration events, but also because it is now also proposed that the immunological T (and likely B) cell exhaustion is weaker in recently infected (with shorter exposure to high amount of HBsAg), and youngest, patients (Le Bert et al., 2020). In the case of HIV, even the youngest individuals who are diagnosed positive are immediately treated with DAAs.

Regarding now the design and implementation of novel combination therapies including novel types of drugs (e.g. RNAi, CpAMs, ASOs, etc.), what could be the final words? The aim to cure HBV infections while avoiding long-life treatment is meaningful; finite-duration regimens are awaited/expected by patients. But to avoid frustrations and failures in future clinical evaluations, as brilliantly discussed in this very recent opinion paper (Pawlotsky, 2022), expectations have to be redefined, as well as therapeutic end-points. Regarding the latter it is clear that we have to define virological end points, but in the mean time we should not forget about clinical end-points (fibrosis, cirrhosis, HCC

occurrences); an improved combination therapy (featuring a NA + a CpAM for instance), in terms of viro-suppression (even absence of HBsAg seroclearance), may prove useful to reduce further the incidence of HCC in patients, even if it is not a finite duration regimen; in this respect one could hope that a CpAM or a RNAi will make it to the clinic to increase the number of types of molecule in our arsenal. An increased rate of and durable HBsAg seroclearance (ideally associated with an anti-HBs seroconversion and recovery of T/B cell functions; this latter recovery of immune functions may require an immunological “kick”) remain a major objective, as HBsAg loss is clearly associated with a decreased (yet not annihilation) of HCC incidence (Diao et al., 2022b; Jin et al., 2022; Yang et al., 2022; Yip et al., 2022); but do we need to decide that such an HBsAg seroclearance has to be obtained in no more than 48 weeks? Surely not! Longer-term, yet with finite duration, regimens might be necessary, and this has to be taken into considerations by R&Ders, stakeholders, and funding supports. A schematic “roadmap” for this R&D is given in Fig. 3. The regulatory path for drug development will also need to evolve (as done for HIV and HCV) to allow a faster evaluation of new compounds and their combinations and an efficient translation to clinical applications towards a cure of HBV infection.

Declaration of competing interest

DD received, in the last 10 years, research grants from Arbutus Biopharma, Gilead Sciences, Janssen, and Enyo Pharma (*on-going*). He has also “interacted” with pharmaceuticals companies (*i.e.* Gilead Sciences, Janssen, Evotec, ImmuneMed, RDP Pharma AG) by running “Fees for Services” contracts.

Data availability

No data was used for the research described in the article.

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