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Antivirals interacting with hepatitis B virus core protein and core mutations may misdirect capsid assembly in a similar fashion

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Abstract

Recently, heteroarylpyrimidines (HAP) have been identified as potent inhibitors of capsid maturation. Here we discuss the HAP mode of action comparing the aggregation phenotype of wild-type and mutant core proteins with the respective phenotype imposed by HAP or other agents interacting with core protein. Pertinent tests include core fusion protein-mediated transactivation in a two-hybrid system and capsid formation. The finding that transactivation appeared to be unaffected by HAP, or by mutations preventing assembly, is surprising and raises the question for the structure of the interacting hybrid core proteins: Are they monomers, dimers or even oligomers? A direct activity of core fusion monomers is not excluded but considered to be highly unlikely due to rapid homodimerisation. A role of core fusion dimers in transactivation would indicate distinct interactions with a differential sensitivity to HAP. Regarding significance of data gained in two-hybrid systems, caution is necessary, since the site of transactivation is the nucleus, whereas the real site of the core protein interactions during replication is the cytoplasm. Apparently, HAP leave the monomer–monomer interface of HBV core protein unaffected but prevent capsid maturation by interacting with a region known to be crucial for dimer multimerisation and formation of stable capsids. It is suggested to use antivirals as tools for the elucidation of early steps in genome replication and capsid assembly. A frame for this could be the hypothesis that the virus uses soluble core protein, namely intracellular maturation intermediates of HbeAg for a core targeted self-restriction of replication.

Keywords: Antivirals; HBV core protein; Misdirection; Capsid assembly; Mammalian two-hybrid system

1. Introduction

HBV infection is a global medical problem. More than 400 million are chronically infected with the major complications being development of liver cirrhosis and hepatocellular carcinoma which is estimated to cause about 1 million deaths annually [1]. In the infected hepatocyte, a covalently closed circular DNA serves as template for synthesis of genomic and subgenomic transcripts. Of the two genomic RNAs, one functions as pregenome, mRNA for the core protein of the virus, and mRNA for the viral polymerase. The second genomic RNA serves as mRNA for precore protein, the precursor of HbeAg, a soluble derivative of core protein secreted by infected hepatocytes that supporting replicative processes. Genome replication depends on the assembly of viral core protein into nucleocapsids (in the following designated as capsids) encasing the RNA pregenome together with the polymerase. Thus, capsids provide the structural background for pregenome reverse transcription into viral DNA. In cell culture experiments, HBV replication can be induced by DNA constructs driving the transcription of RNA pregenome.

Mutations affecting core gene expression cause replication deficiency but can be complemented by coexpression of an intact core gene [2]. When expressed alone, i.e. under non-replicative conditions, core protein assembles into ‘‘empty’’ capsids. The HBV core protein consists of 183 amino acids and is present in its non-particulate form as dimers that are considered to be precursors of capsid maturation. The prevailing opinion is that capsids are formed in a rapid process directly from dimers without discrete intermediates when a critical dimer concentration is exceeded [3–5]. Initiation and maintenance of capsid formation leads to a constant dimer concentration [6].

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2. Are dimer-oligomers intermediates in the process of capsid maturation?

Electron microscopy and velocity sedimentation studies on HBV capsid formation failed to detect higher-order multimer intermediates as found for capsid assembly in other viruses [7]. Recently, based on *in vitro* studies on the assembly of purified core protein, a hypothesis modifying this view has been proposed. Accordingly, capsid assembly is nucleated by a trimer of dimers and advanced via the addition of individual dimers [8,9]. Following nucleation, weak protein–protein interactions appeared to be sufficient for driving assembly.

Aside from the hypothetical nucleation trimers, there are only a few reports on core oligomers. A multimeric particle has been described by Lingappa *et al.* [10] associated with a chaperonin-like protein, which may assist capsid assembly but is neither complexed with dimers nor with mature capsids. In addition, a heteromeric complex referred to as ''pre-assembly complex'' composed of HBV pregenomic RNA, polymerase and cellular factors is generally assumed to initiate capsid assembly by recruiting core protein [11,12].

Regarding the molecular basis for inter-dimer interactions, the core regions between 113aa and 143aa possibly including 141 and 149 are crucial [13,14]. If interfering mutations are introduced into this region, unstable capsids are formed or their assembly is totally prevented [13], suggesting that the above regions are the main contributors to multimerization.

3. Core protein interactions in mammalian two hybrid systems

In addition to *in vitro* assembly and to assembly observed in cell culture systems, core protein interactions have been studied using a mammalian two-hybrid system [15]. In this system [16], the reacting HBV core protein (183aa) is fused to the GAL4 DNA binding domain (147aa) resulting in GAL-core, and to the VP16 transactivation domain (46aa) resulting in VP16-core hybrid protein, respectively. Despite their fusion protein nature, they reproduced aspects of core protein interaction. It is open, however, whether specifically the core monomer interaction, i.e. dimerisation can also be assessed.

Apart from the propensity of the core protein to dimerize by itself—the formation of GAL-core dimers is further predetermined, since the GAL4 domain binds to DNA as a dimer [17]. Based on the strong *cis*-preference of dimerisation which has been recognized upon co-expression of discernable core proteins, Chang *et al.* [15] suggested that any activity detected in the two-hybrid system involves interactions of core protein dimers, namely dimerized GAL-core with dimerized VP16-core rather than interactions of the respective monomers. The authors even conceived a cotranslational dimer formation between adjacent, nascent core polypeptides still connected to the translating ribosomes as this has directly been shown for p53 [18]. The rapid dimerisation of nascent core polypeptides would explain the absence of monomers in the oocyte capsid assembly system [3]. However, a post-translational formation of hetero-dimers consisting of one GAL-core and one VP16-core cannot be excluded.

Regarding the critical assessment of transactivation data obtained for core–core interactions in the two-hybrid system, it should be kept in mind that both the GAL-core and VP16-core fusion proteins contain a non-HBV nuclear localisation signal (NLS) ensuring a sufficient high transport to the reporter gene in the nucleus. In the case of the 183aa core protein an additional viral NLS consisting of two direct repeats in the carboxyl terminal region is present [19,20] which may affect nuclear transport and thereby transactivation. The activity of this viral NLS, however, is suggested to depend on 10 amino acids present only on the N-terminus of the precore protein and HbeAg [21].

4. Capsid assembly as a target for antivirals

At present, there is no generally effective therapy for chronic hepatitis B [22]. Most of the present clinical regiments for treatment such as nucleoside analogues and/or interferon have limited success [22,23]. Reasons for this are drug resistance due to development of polymerase mutants, and the limited efficacy of interferon treatment with a sustained response of only 25–40% of patients selected for trials [23].

Novel experimental approaches target the HBV core protein to interfere with capsid formation as a process necessary for genome replication and assumed to be less prone to developing drug resistance. The fact that weak core–core interactions are sufficient to drive capsid assembly [9] facilitates an effective targeting. As a further interesting aspect, core assembly may be the natural target for a precore-mediated self-restriction of replication [2,24]. In line with this, the coexpression of precore protein suppresses core protein assembly into empty capsids.[1]

In principle, the level of action of core-specific antivirals could range from prevention of dimerization, interference with the formation of the pre-assembly complex to inhibition of capsid maturation and envelopment. In addition, it is conceivable that these compounds may exert their effects at more than one step. Examples for core-specific antivirals are: Intracellular expression of anti-core single chain antibodies [25], core-specific aptamers [26], iminosugars, though without fully proven anti-core activity and only assumed to prevent packaging [27], and bis-ANS (5,5′-bis[8-(phenylamino)-1-naphtalenesulfonate]) [28]. The inhibition of capsid formation by heteroarylpyrimidines

[1] Ratsch and Schröder, unpublished.

(HAP) in virus producing cells has been described by us in some mechanistic detail as a promising therapeutic approach [29].

5. HAP does not inhibit core protein translation but capsid formation in cultured cells sustaining HBV replicative processes

It has been shown that HAP itself is effective as an HBV antiviral. Several metabolites of HAP have been tested for anti-capsid-formation activity and displayed either less or no activity compared to the non-metabolized parent compound (supporting online material of [29]). At a HAP concentration just sufficient to inhibit capsid formation (0.4 μM; ([29], Fig. 2B)), no reduction in amount of total core protein is observed, supporting the notion that HAP binding to core protein primarily prevents capsid formation while translation is not affected. At higher doses, HAP causes a reduction in core protein levels. This is likely due to a reduction in the core protein overall half-life. Since incorporation of core dimers into capsids protects the protein from degradation, the apparent half-life actually represents the half-life of the non-associated dimer protein [29]. The comparably high stability of core protein measured at HAP doses just sufficient to cause capsid maturation, may indicate the existence of protective non-capsid polymers which are not formed at higher doses.

6. Binding of HAP to core protein

Although clearly targeted to the viral core protein—core assembly is inhibited even when expressed under non-replicative conditions (supporting online material text S5 [29])—the precise site of core protein–HAP interaction and precise step of the replication cycle at which HAP primarily interferes is not known. Since HAP molecules do not carry reactive groups, covalent binding to core protein can be excluded, rather, an interaction is brought about by hydrophobic and polar forces between drug and protein. So far, a direct binding of HAP has only been shown on *E. coli* expressed capsids. It was restricted to the (−)-*R*-enantiomers which also blocked capsid assembly [29]. About 100 molecules of ^{3}H-labeled HAP per capsid were calculated assuming a structural homogeneity of the preparation. Following agarose gel electrophoresis verifying the presence of capsids, only about one molecule HAP per capsid was found, suggesting if at all, an unstable binding of HAP to capsids under electrophoresis conditions. It cannot be excluded, however, that the *E. coli* capsid preparation used contains core protein oligomers capable to bind HAP, for example diffusible soluble forms and labile aggregates which are difficult to identify. In any case, HAP does not influence the stability of assembled capsids.

For studying interactions preventing capsid assembly, it is a more reasonable approach to start with the reactivity of dimers [28] because, once capsids are formed, interaction with drugs may be strongly reduced or impossible. Therefore, a HAP binding study should start with a dimer solution—under conditions preventing assembly—to which HAP is added. Then conditions are changed to allow capsid assembly. Subsequently, assemblies and bound HAP are determined. Labile non-capsid aggregates following exposure of core dimers to bis-ANS, have been identified by Zlotnick *et al.* [28] using electron microscopy. However, their identification by size exclusion chromatography failed. bis-ANS is a fluorescent probe binding to hydrophobic sites of proteins and is known to prevent tubulin polymerisation [28]. Similar experiments with HAP resulted in the formation of large structures resembling inflated capsids (Fig. 1). Possibly, the huge size of these structures is due to a lasting effect of bound HAP altering the geometry of dimer–dimer interaction (Fig. 1B).

7. Two hybrid transactivation and capsid formation, a two parameter system for testing core protein interactions in the absence and presence of inhibitory drugs

As mentioned above, interacting HBV core fusion proteins studied in the mammalian two-hybrid system [15] are most likely dimeric themselves, i.e. the results of a protein–protein interaction (a GAL-core and a VP16-core homodimer). Despite the more or less bulky non-viral N-terminal moieties, fusion proteins of this type are still capable of forming capsids [30]. Since HAP abrogates dimer–dimer interaction as a prerequisite for capsid formation, one could reason that it should also block dimer-mediated activation of the reporter gene. Whether this is indeed the case was tested on cells of the HuH7 line by the Checkmate® (Promega) mammalian two-hybrid assay which is similar to that used by Chang *et al.* [15], except for luciferase replacing CAT as reporter gene.

Plasmids expressing the fusion proteins were first established as a series of core fusion constructs in the yeast two-hybrid system [26], and then the sequences encoding the following core moieties were transferred to the respective plasmids of the mammalian system: complete 183aa wild-type protein and carboxy terminally truncated 149aa and 124aa core proteins. In addition, a 149aa core protein mutated at position 127 (R → Q) was included.

Both the 149aa (127 Q) mutant and the 124aa proteins form dimers but no capsids [13]. Reporter gene activity and capsid formation were analyzed in cytoplasmic extracts from parallel culture dishes. Agarose gel electrophoresis and direct blotting of core particles were performed as previously described [26].

Following expression of the 183aa core-fusion proteins in HuH7 cells, formation of capsid-like structures was

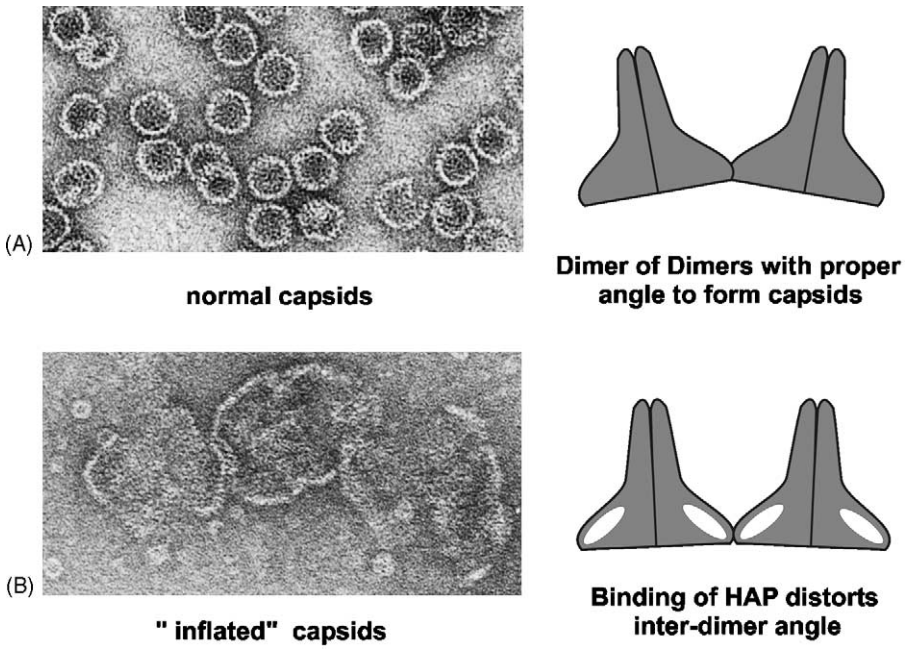

Fig. 1. Assembly of core protein in absence or presence of HAP. Core protein shells (1–149aa) made in bacteria were dissociated into dimers using urea/high pH, purified and then re-assembled by dialysis at low pH, either in the absence, or in the presence of HAP. Subsequently, capsids were processed for electron microscopy and photographed under the same magnification. In the absence of the compound, (A), compact spherical capsids are formed with an average diameter of 30 nm. However, in the presence of the compound, (B), large irregular complexes are formed resembling ''inflated'' capsids.

observed in all cases (Fig. 2). They migrated slower than capsids formed by the 183 wild-type protein (not shown). As expected, upon co-expression of the 183aa pair, an increase in the luciferase reporter gene activity (Fig. 2, ACT + BIND) was observed. Similar data were gained for the 149aa pair, except for a lack of assembly when VP16-149aa fusion protein (ACT) was individually expressed. Apparently, in the case of this truncated core protein species, the VP16-domain (46aa) disturbs assembly in contrast to the much larger GAL4-domain (147aa). The migration behavior of capsid-like structures upon co-expression is different from those formed individually by the GAL-core fusion protein. This observation clearly indicates a substantial structural contribution of the VP16-core protein (Fig. 2, core moiety 183, compare position of ACT capsids with those of BIND and BIND + ACT).

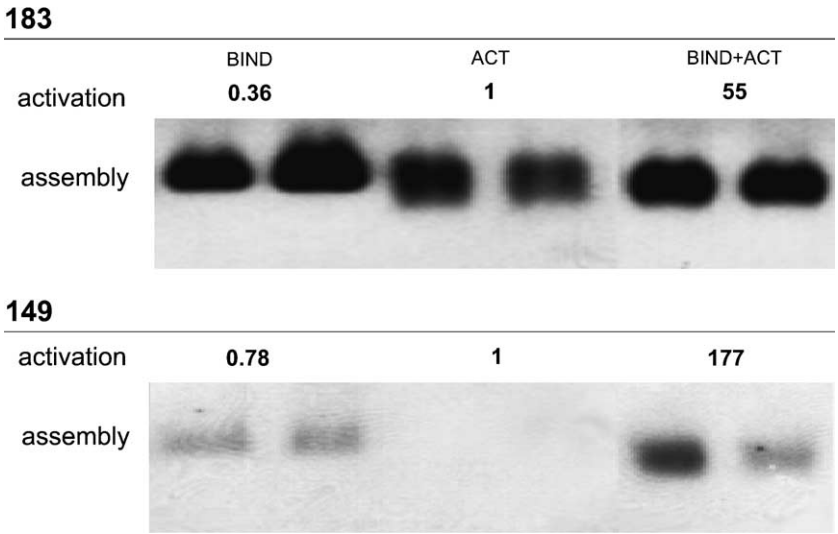

Fig. 2. Transactivation and core assembly in the mammalian two-hybrid system. Sub-confluent HuH7 cells (5×10^5 per 60 mm dish) transfected according to the method of Chen and Okayama [35] with 2 μg of the pG5*luc* reporter plasmid and the following core fusion constructs: VP16-183 (ACT), and the GAL-183 (BIND), and the respective 149 constructs, were harvested 2 days post-transfection. The amount of core-fusion plasmid constructs was 5 μg. Capsids were analyzed as reported by Butz *et al*. [26]. Firefly-luciferase activity (reporter) was assayed as described earlier by Hilger *et al.* [36]. Data are the mean values acquired from duplicate experiments and determinations.

8. In the two-hybrid assay, HAP does not inhibit transactivation but blocks assembly of capsids

HAP did not affect transactivation by the ACT and BIND core-fusion pairs 183aa and 149aa but inhibited their assembly. Thereby, the assembly-negative phenotype of the construct pairs 149aa (127Q) and 124aa observed in the absence of HAP is mimicked (Fig. 3). In line with this, the transactivation capacity displayed by these latter constructs was insensitive to HAP (Fig. 3).

It is an attractive idea that the structural alterations imposed by mutation and truncation, respectively, are comparable to those induced by HAP. A most probable target of HAP action then would be a core protein region considered to direct multimerisation which has been designated as region II (aa position 113–143) by König *et al.* [13]. It is noteworthy that the core-specific peptide aptamer C1-1 reported by Butz *et al.* [26] to inhibit capsid formation appears to be targeted to this same region.

Accepting that the core–core interaction studied in the mammalian two-hybrid system is an interaction between dimer proteins [15], then the dimer interaction leading to transactivation of the reporter gene may indicate an early step in the pathway of capsid formation that cannot be disrupted by HAP, or an aberrant pathway of aggregation induced by HAP. The capacity of mutated core constructs to form labile aggregates [13] supports the possibility that HAP may inhibit capsid formation but still allow aggregation of dimers to non-capsid polymers as observed for assembly in the presence of bis-ANS [28].

It is surprising that interference with capsid formation apparently does not change the amount of core fusion protein available for transactivation. Possibly, transactivating oligomers originate from interactions of free dimers that are present at a sustained threshold concentration beyond which polymerisation and assembly are commenced. Two types of non-capsid core-dimer aggregates could coexist in the two-hybrid system in relation to transactivation, active ones (oligomers) and inactive ones (polymers including capsids).

Capsid formation in the two-hybrid system may actually reflect *in vivo* assembly as it is observed in the cytoplasm of hepatocytes sustaining HBV replicative processes. However, for the oligomers active as transactivators in

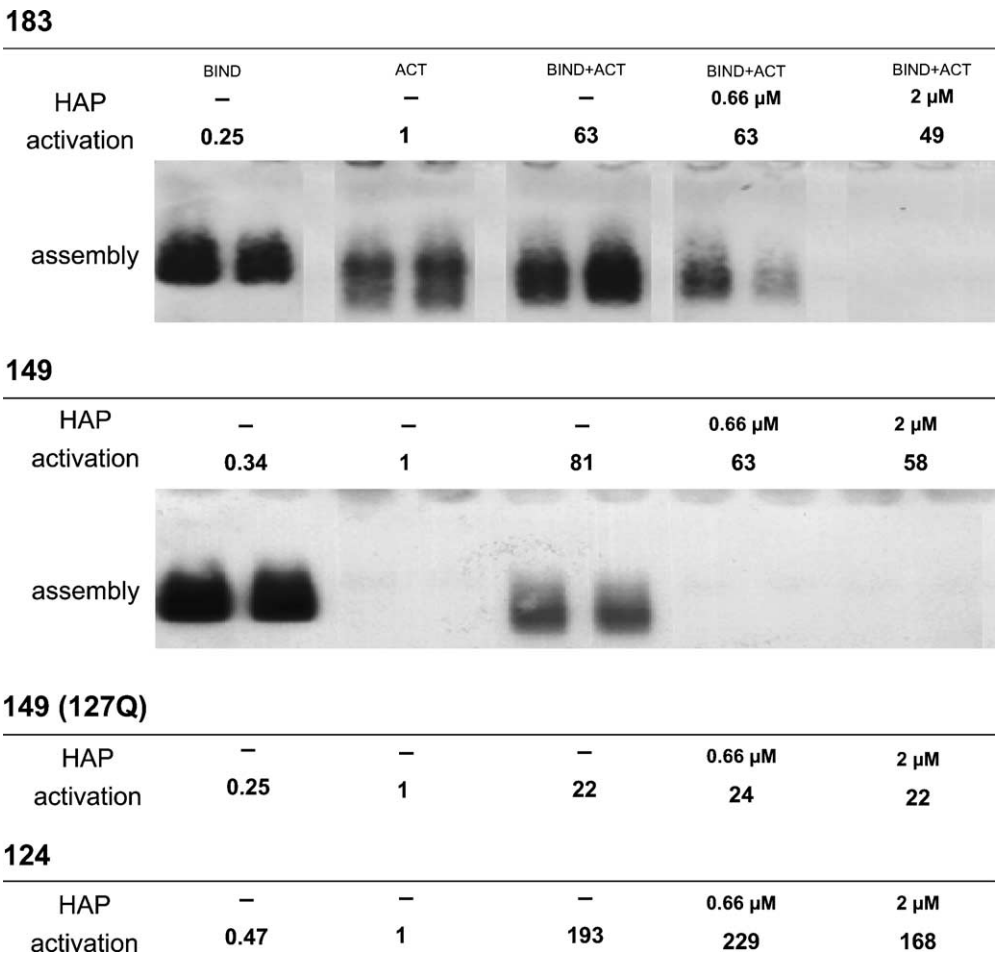

Fig. 3. The parameter transactivation is insensitive to HAP. Transactivation and core assembly in the two-hybrid system studied in the absence and presence of 0.66 and 2 µM of the HAP-congener Bay 41–109 [29]. HAP was added together with the transfecting DNA and re-added after the washing step in the transfection procedure (see Fig. 2).

the two-hybrid system, it cannot be excluded that their formation is influenced by the artificially introduced non-HBV nuclear localisation signal and the specific conditions in the nucleus. For example, the fraction of soluble core protein could be higher in the nucleus than in the cytoplasm due to an efficient nuclear transport. Transactivating aggregates could be directly generated in the nucleus or preformed in the cytoplasm followed by nuclear import. The transport of capsids across the nuclear membrane or assembly in the nucleus are principally possible [31,32], yet the size of capsids should relate to a low specific activity or even a total lack of a capacity to activate the reporter gene.

9. Future research on core-specific antivirals (screening and identification of target sites)

Future work should focus on establishing systems detecting core-specific drugs, either modified ones currently under study, or new ones. Clearly, these activities require a combined effort of laboratories specialised in high through-put test systems and others involved in more basic aspects of the HBV life cycle. Specifically, research on the disruption of capsid formation should be continued to characterise non-capsid polymers and to identify crucial steps accessible to drug action. Expected is a more refined picture of particle maturation which provides a broader basis for the design of specific anti-HBV drugs.

10. Screening for substances (small molecules) preventing transactivation in the mammalian two-hybrid system

Interference with interaction between core dimers rather than with complementation of core oligomers to full capsids by dimer addition can be considered as an early, and probably the most effective step to block capsid assembly. It should be tested whether drugs known to directly interact with core protein such as bis-ANS and HAP can be modified in a way that they inhibit dimer–dimer interaction in the mammalian two-hybrid system. The question is, however, whether it is possible to confine core protein interaction to the mere formation of dimers. Furthermore, new non-nucleosidic antivirals, single chain antibodies and aptamers could be screened for similar inhibitory patterns. Aptamers effective in the two-hybrid system, could provide in a second step a basis for the design of mimetic drugs [33] with anti-HBV activity. As a technical approach of potential value, a modified two-hybrid system could be used to study dissociation rather than interaction of proteins. So far, this is only possible in yeast using the so called reverse two-hybrid system [34]. Initial studies could be carried out in yeast and used for the development of an equivalent mammalian system.

11. Identification of target sites critical for core protein interaction and assembly intermediates

For a better understanding of the HAP mode of action at the level of dimers, the precise binding site—the core region 113–143aa being a prime candidate—and the resulting changes in core protein conformation affecting dimer-interaction should be studied. The possible interaction of HAP with the formation of the putative HBV pre-assembly complex consisting of the RNA pregenome and the polymerase as the natural nucleation center for particle maturation should be included, yet would require its identification.

The prevention of formation of genuine capsids conceivably could lead to the accumulation of natural intermediates of capsid maturation. Despite the generation of artificial non-capsid polymers, such intermediates like trimeric dimers, the chaperonine-like protein containing core complex, and the putative pre-assembly complex may still become accessible to analysis. For a description of such intermediates it may be helpful to know the precise interaction sites on core and precore protein which provide the basis for the natural self-limitation of HBV replication. Obtained data for the drug/core protein and core/precore interaction can be expected to complement each other in the elucidation of crucial steps in particle maturation.

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