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A fluorescent analog of a HBV core protein-directed drug is used to interrogate antiviral mechanism

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Abstract

Heteroaryldihydropyrimidines (HAPs) are antiviral small molecules that enhance assembly of HBV core protein (Cp), lead to assembly of empty and defective particles, and suppress viral replication. These Core protein Allosteric Modulators (CpAMs) bind to the pocket at the interface between two Cp dimers and strengthen interdimer interactions. To investigate CpAM mechanism, we wanted to examine the cellular distributions of Cp and the CpAM itself. For this reason, we developed a fluorescently labeled CpAM, HAP-ALEX. *In vitro*, HAP-ALEX modulated assembly of purified Cp and at saturating concentrations induced formation of large structures. HAP-ALEX bound capsids and not dimers, making it a capsid specific molecular tag. HAP-ALEX labeled HBV in transfected cells, with no detectable background with a HAP-insensitive Cp mutant. HAP-ALEX caused redistribution of Cp in a dose dependent manner consistent with its 0.7 μ M EC₅₀, leading to formation of large puncta and an exclusively cytoplasmic distribution. HAP-ALEX co-localized with the redistributed Cp but large puncta accumulated long before they appeared saturated with the fluorescent CpAM. CpAMs affect HBV assembly and localization; with a fluorescent CpAM both drug and target can be identified.

Introduction

Hepatitis B virus infection can cause chronic or acute hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 2 billion people are globally estimated to be infected with HBV, of these over 270 million are chronic carriers.^{1,2} Claiming over 750,000 lives a year, with 50% of this number attributed to liver cancer alone, HBV infection is ranked as one of the leading causes of human mortality.³ Current treatments include interferon therapy and nucleotide analogs or a combination of both.^{4–8} However, the efficacies of these therapies are limited owing to issues like persistent covalently closed circular DNA (cccDNA), emergence of drug resistant viruses, and that interferon is most effective in only a subset of chronic patients.^{4–10} This necessitates a more robust therapy curbing HBV at multiple steps of its life cycle.

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HBV is an enveloped virus, with a partially double stranded DNA genome that replicates via an RNA intermediate¹¹. The viral core is comprised of an icosahedral protein shell made up of 120 core protein (Cp) dimers (Figure 1A), the partially double stranded DNA genome, the viral polymerase, and necessary and serendipitous host proteins, from chaperones to restriction factors.^{12–16} Newly translated core proteins assemble in the cytoplasm encapsidating the polymerase and pre-genomic RNA-polymerase complex and provide the microenvironment for reverse transcription of pre-genomic RNA (pgRNA) to synthesize the relaxed circular DNA (rcDNA) of mature cores. The core protein is also involved in trafficking of the viral genome (rcDNA) into the nucleus to make the cccDNA^{17,18} and interacts with the envelope protein required for viral secretion.¹⁹ Thus, HBV core protein assembly is keystone of successful HBV infection that provides a structural framework to the virus. Cp is usually localized to the cell nucleus of patients with chronic hepatitis with minimal liver damage and is cytoplasmic in patients with active hepatitis^{20–27}. Cp is distributed both in cytoplasm and nucleus in HBV-producing primary hepatocytes and transgenic mice.^{20,28–31}

HBV Cp is comprised of a 149 residue assembly domain and 34 residue C-terminal RNA binding domain that also serves as the nuclear localization signal.³² The Cp149 assembly domain is sufficient for assembly into capsids that are indistinguishable from wild type capsids.³³ The dimer comprises a four-helix bundle, with each monomer contributing a pair of helices (Figure 1B). The tetravalent dimers self-associate to form stable capsids based on a network of weak dimer-dimer interactions. The weak contact energies allow “proof-reading” to ensure high fidelity assembly. Conditions that favor stronger association energies and accelerate assembly kinetics have a notable tendency to produce kinetically trapped defective particles.³⁴

Small molecules, with a variety of chemical scaffolds, have been discovered that can enhance HBV Cp assembly by favoring an assembly active form of Cp. Collectively these are called Core protein Allosteric Modulators (CpAMs). These include heteroaryldihydropyrimidines (HAPs), phenylpropenamides and sulfamoylbenzamides.^{35–37} These molecules bind a pocket at the interface between core protein dimers and strengthen the dimer-dimer associations. *In vitro*, saturating concentrations of HAPs induce assembly of aberrant structures. *In vivo*, HAP treatment leads to suppression of production of RNA-filled immature capsids and mature virions,^{35,38,39} presumably due to competition between CpAM induced and polymerase-pgRNA-induced nucleation.

HAP specificity towards HBV CP makes them a perfect candidate for molecular tagging and for tracking core protein in cells. With this aim, we designed and synthesized a fluorescent HAP derivative. We chose Alexafluor 488 because of its utility in fluorescence microscopy. We tested this fluorescent HAP (HAP-ALEX) for its effect on HBV assembly *in vitro* and its efficacy to act as an *in vivo* HBV label/tag. We find that *in vitro*, HAP-ALEX, like other HAPs, is a highly active assembly effector that induces large assembled structures at higher concentrations. Interestingly, HAP-ALEX bound capsids and not dimers, making it a capsid specific molecular tag. Likewise, HAP-ALEX exhibited antiviral activity and labeled HBV capsids in transfected cells, however it caused redistribution of Cp in a time and dose dependent manner.

Materials and Methods

Design and Synthesis of Fluorescent HAPS

HAP-ALEX was synthesized from HAP13 as previously described using ALEXA 488 succinimidyl ester (Thermo-Fisher) for installation of the fluorophore.^{40,41} Stocks of HAP-ALEX were dissolved in DMSO and diluted into an aqueous buffer, HEPES or DMEM cell culture media, prior to use.

Purification of Cp149 and Assembly assay

The assembly domain of HBV Core protein was overexpressed and purified in its dimeric form as previously described.⁴² Cp149 assembly was monitored by 90° light scattering using a fluorometer with excitation and emission monochromators set to 400 nm to minimize absorbance from the HAP-ALEX. Purified Cp149 dimer (10 μ M dimer in 50 mM HEPES buffer) was incubated with increasing concentrations of HAP-ALEX or HAP13 for 1 min at 25°C. Assembly was initiated by adding equal volume of 50 mM HEPES, 300 mM NaCl to attain a final concentration of 5 μ M Cp149 dimers in 50 mM HEPES and 150 mM NaCl.

Electron Microscopy (EM)

Assembly reactions products obtained following the light scattering experiments were examined by negative stain transmission electron microscopy (TEM). Briefly, 4 μ L of assembly product was applied to a glow discharged carbon-coated grid (Electron Microscopy Sciences) and incubated for 30 seconds. The grid was blotted, rinsed with water and stained with 2% uranyl acetate and air dried. Grids were imaged with a JEOL-1010 transmission electron microscope equipped with a 1k x 1k Gatan CCD camera (MegaScan model 794) (stationed at IU Bloomington Electron Microscopy Center).

Size Exclusion Chromatography

Assembly reactions were examined by size exclusion chromatography on a Superose6 (GE Healthcare) column mounted on a Shimadzu HPLC. For this ~24 ml column, capsid usually elutes slightly after the ~8 ml void volume while dimer elutes around 17 ml. The column was equilibrated with 50 mM HEPES and 150 mM NaCl. Free HAP-ALEX elutes at the end of the chromatograph in an ensemble of peaks which suggests weak adsorption to the column. Absorbance was measured at 280 nm for protein and HAP-ALEX and 495nm for HAP-ALEX alone.

Cell Culture, Transfection, Western blots, and HAP labeling

HuH7-H1 cells (a kind gift from Daniel Loeb, U. Wisconsin) were maintained in DMEM-F12 (Gibco) media supplemented with 5% FBS (Gibco), 1x Antibiotic Antimycotic (Sigma), 600 μ g/ml of G418 (Clontech). HBV genomic clones used in the study are LJ144 (an HBV expression plasmid that does not produce the viral envelope or surface protein); LJ144-V124W (HAP-insensitive mutant of LJ144), Y63F (polymerase mutant clone); Y63F-V124W (HAP insensitive mutant of Y63F) and pTruf-HBc (Cp clone). Both Y63F clones are also envelope protein deficient. HuH7-H1 cells seeded on 6 well plate were transfected with 750 ng of HBV expression plasmids using TRANSIT-LT1 (MIRUSBio) transfection

reagent. 3 days post transfection the cells were trypsinized and re-seeded on coverslips in a 24 well plate. 6 hours after seeding, cells were treated with 1 μ M HAP-ALEX for 16 hours.

For the HAP-ALEX time course and concentration studies, cells were seeded on a coverslip in 24 well plate and transfected with 250 ng of plasmid DNA. 24 hours post-transfection, cells were treated with 1 μ M HAP-ALEX for 2, 4, 6, 8, 16 or 24 hours. Alternatively, 24 hours post-transfection, cells were treated with 0.1, 0.2, 0.5, 1 or 2 μ M HAP-ALEX for 16 hours. To test for changes in CP concentration in response to HAP treatment, western blots were performed as previously described.¹⁵

Measuring EC50 and cell toxicity

Antiviral activity of HAP-ALEX was assayed in virus producing HepG2.2.15 cells. Cells were seeded in 96 well plates in sets of two triplicates- one set for EC50 measurement and another for cell toxicity. Two days post seeding, culture media was changed, and cells were treated with different concentrations of HAP-ALEX (5nM-10 μ M) for five days. It takes 5 days for HepG2.2.15 cells to accumulate enough virus and core protein to be quantified without ambiguity under drug repression conditions. A metabolically labile drug will show weaker antiviral activity and higher background signal. As HepG2.2.15 is not an inducible expression system, so there will always be some background. DMSO treated cells served as untreated controls. For EC50 measurements culture media was collected 5 days post treatment and HBV DNA was extracted using Nucleospin Blood kit (Macherey-Nagel) as per the manufacturer's protocol. 2 μ l of this DNA was used to quantify the HBV genome equivalents by qPCR using Luna[®] Universal qPCR Master Mix (NEB) on an Applied Biosystems StepOnePlus[®] real time PCR system. A standard curve was obtained with LJ144 plasmid- a HBV genomic clone. The data was analyzed using Applied Biosystems StepOnePlus[®] software. Cell viability was measured by addition of 100 μ l of CellTiter-Glo[®] 2.0 reagent (Promega) directly to wells. Cells were lysed for 2 mins on an orbital shaker and followed by a 10 mins incubation at room temperature to stabilize the luminescent signal. The contents were transferred to opaque flat bottom 96 well plate (Greiner Bio-one) and luminescence was measured using a Bio-Tek Synergy H1 plate reader.

Immunofluorescence (IF) and Confocal Microscopy

Following HAP-ALEX treatment, cells were washed thrice with 1x PBS and fixed with 10% formaldehyde. Cells were permeabilized with 0.2% Triton X-100 and blocked with 2% BSA. The cells were incubated with 1:500 dilution of a polyclonal rabbit anti-Cp primary antibody (Dako) for 4 hours at room temperature (RT) followed by incubation with 1:500 dilution of goat anti-rabbit secondary antibody, Alexa Fluor 594 conjugated (ThermoFisher Scientific) for 2 hours at RT. Coverslips were treated with a drop of VECTASHIELD Antifade mounting media containing DAPI (Vector Laboratories) and mounted on glass slides. Coverslips were sealed with clear nail polish before imaging.

Signals from HAP-ALEX and Cp immunofluorescence (Alexa 594) were visualized using a Leica TCS SP8 confocal microscope equipped with Leica HyD Detector (IU Bloomington Light Microscopy Imaging Center). A 63X (f1.4 numerical aperture) oil immersion objective (Leica) and Argon Laser (at 488 nm and 594 nm) was used for imaging in the "Line-

Sequential” mode of the LAX Image software. Images were analyzed using Fiji IMAGEJ software (<https://fiji.sc/>).

pgRNA Quantification

HuH7-H1 cells seeded on 6 well plate were transfected with 750 ng of HBV Y63F expression plasmid using TRANSIT-LT1 (MIRUSBio) transfection reagent. 3 days post transfection the cells were treated with DMSO or 1 μ M HAP-ALEX for 16 hours. Cells were lysed in lysis buffer (50 mM HEPES pH7.4, 1% NP40, protease inhibitor tablet (Roche)). Cell lysates were treated twice with 60U of Micrococcal Nuclease (NEB) in presence of 5mM CaCl₂ for 30 mins at 37°C to remove all non-core protected nucleic acid. Total RNA was extracted from these nuclease treated cell lysates using RNAeasy kit (Qiagen). 2 μ l of this RNA was used to quantify the pgRNA by quantitative RT-PCR using Luna[®] One Step qRT-PCR Master Mix (NEB) on an Applied Biosystems StepOnePlus[®] real time PCR system. A standard curve was obtained using purified *in vitro* transcribed pgRNA.

Results

Design and Synthesis of Fluorescent HAP-ALEX

Design of HAP-ALEX was based on the co-crystal structure of a HAP molecule (Figure 1C) in complex with an HBV capsid (Figure 1A).^{43,44} The HAP moiety was located at the interface between two dimers and with position 6 of the dihydropyrimidyl ring pointed towards the interior of the capsid. Structure activity analysis showed that polar extension of HAPs from the position 6 was well-tolerated.⁴⁴ To generate a fluorescently-labeled HAP, a piperazine at position 6 (Figure 1C, Compound 1) was extended by a linker to a HAP-ALEX fluorophore (Figure 1C, Compound 2). The proposed model of the complex is consistent with a recent cryo-EM structure of a HAP-TAMRA derivative.⁴⁰ Details of HAP-ALEX characterization are included as Supplementary Information Figure 1.

Effect of HAP-ALEX on assembly

To functionally act as an *in vivo* label for HBV core protein, HAP-ALEX must bind core protein. We hypothesized that interaction *in vitro* would provide a guide to HAP-ALEX efficacy *in vivo*. We tested HAP-ALEX for its ability to modulate Cp149 assembly and bind to Cp149 polymers.

Assembly kinetics were monitored by light scattering. The 35 kDa dimer scatters much less light than the 4 MDa capsid; the effect is even greater with the very large complexes observed when Cp assembly is induced by high concentrations of HAP molecules.⁴⁵ For these studies we examined light scattering at 400 nm, a relatively short wavelength to maximize signal with minimal absorbance by HAP-ALEX and protein. Cp149 assembly is sensitive to ionic strength. At 150 mM NaCl, the pseudo-critical concentration of assembly is about 10 μ M dimer, therefore any increase in light scattering is due to HAP-ALEX-induced assembly. Keeping in mind that HAP-ALEX is a racemic mixture and that only one isomer is active,⁴⁶ Cp149 assembly kinetics were noticeably stimulated at a sub-stoichiometric concentration of only 1.25 μ M HAP-ALEX, a ratio of eight dimers per active CpAM. At concentrations $\geq$ 5 μ M, HAP-ALEX-induced assembly products scattered more

light than the capsid control formed from 5 μ M dimer (Figure 2A). This excess light scattering demonstrates that the fluorescently tagged HAP molecule can enhance HBV assembly and at higher concentrations induce large complex formations, also evident in negative stain electron micrographs of assembly products (Figure 2B). Strikingly, under same assembly conditions parent HAP13 drove assembly sluggishly, exhibiting slower kinetics and requiring higher concentrations than HAP-ALEX to achieve the same level of assembly (Figure 2A).

We further examined HAP-ALEX-induced assembly products by size exclusion chromatography of assembly reactions after a 24 hour incubation. Even at the lowest HAP-ALEX concentration tested, absorbance at 280 nm (where the protein absorbance is dominant) revealed a decrease in the amount of free Cp dimer. With increasing concentrations of HAP-ALEX there was a concomitant buildup of the capsid peak (Figure 3A, B) in agreement with the light scattering experiment where we also observed that HAP-ALEX strengthened protein-protein interactions. As HAP-ALEX concentration increased, the capsid peak progressively shifted towards the void volume, suggestive of the formation of large non-capsid polymers. Using the ALEXA488 absorbance at 495 nm, we observe that HAP-ALEX co-migrates with the capsid/polymer peak but not with the dimer peak (Figure 3B). Based on the absorbance at 280 nm and 495 nm, we calculated the ratio of HAP-ALEX to Cp dimers and observe that it asymptotically approaches 1:1 based on our structural understanding of HAP binding (Figure 3C).

***In vivo* labeling of HBV intracellular cores with HAP-ALEX**

We tested the ability of HAP-ALEX to bind HBV cores *in vivo* and function as viral tracker. This activity requires the 1043 Da molecule to cross the cell membrane. HuH7-H1 cells were transfected with a HBV genomic clone defective in the envelope protein expression so that viral cores would accumulate in the cytoplasm.⁴⁷ Transfected cells growing on coverslips were treated with 1 μ M HAP-ALEX for 16 hours prior to fixation, permeabilization, and immunostaining. As seen in Figure 4B, HAP-ALEX signal localized in the cytoplasm forming distinct large puncta. Consistently, immunolabeling of Cp in HAP-ALEX-treated cells also showed punctate structures that localized in the cytoplasm and overlapped well, although not perfectly, with the HAP-ALEX signal. Since the anti-Cp polyclonal antibodies we used can detect Cp monomers in a western analysis, it is likely that they were also detecting dimers in cells. Hence, we also tested monoclonal antibody Mab3120 (Institute of Immunology, Tokyo), which has a capsid-specific conformation epitope.⁴⁸ However, HAP-ALEX and Mab3120 mirrored a pattern similar to that with the Dako polyclonal antibody (Figure 4C).

To rule out signal from non-specific binding of HAP-ALEX in the cell, we expressed the HBV core protein mutant V124W. In this mutant, the tryptophan side chain partially fills the HAP pocket and blocks HAP binding.⁴⁷ As predicted for specific interaction, we did not detect any signal from HAP-ALEX, although immunolabeling confirmed the expression of intracellular V124W cores (Figure 4D). V124W mutant cores, which failed to bind HAP-ALEX *in vivo*, had the same localization pattern as wild type Cp in the absence of HAP-ALEX: tiny puncta were observed uniformly distributed mainly in the cytoplasm. These

results show that at a concentration of 1 μM , HAP-ALEX can alter cellular distribution of susceptible core protein (Figures 4B, 4C) while HAP-resistant core protein (Figure 4D) has the same pattern found in DMSO treated control cells (Figure 4A).

This effect is consistent with an antiviral activity for our labeled HAP. We note that the parent compound, HAP13, had a relatively poor EC_{50} of 6 μM in HepG2.2.15 cells⁴⁹. In contrast, we observed that HAP-ALEX had an EC_{50} of $0.71 \pm 0.05 \mu\text{M}$ in the same cell line (Supplementary Figure 2A). EC_{50} values are reported for the racemic mixture, the concentration of active HAP is half the EC_{50} . At 1 μM HAP-ALEX, the concentration used in the immunofluorescence (IF) experiments of Figure 4, HBV production was suppressed by almost 60% in the EC_{50} experiments. HAP-ALEX was non-toxic to cells at up to 10 μM (Supplementary Figure 2B).

Because we found treatment with 1 μM HAP-ALEX for 16 hours led to unambiguous staining and dramatic redistribution of Cp, we next tested the effects of time and dose on activity. Using an expression system that is surface protein deficient (HBsAg^-) (also used in Figure 4A-D), at 24 hours post transfection cells were treated with 1 μM HAP-ALEX for different durations of time. No Cp redistribution was detected 2 hours post treatment by immunofluorescence or by HAP-ALEX fluorescence (data not shown). As early as 4 hours post HAP-ALEX treatment, immunofluorescence shows Cp forming punctate structures (Figure 5A) although at this early time point the HAP-ALEX signal was weak and had little overlap with the antibody signal. HAP-ALEX-induced Cp structures increased both in size and fluorescence with longer treatment (8, 16 and 24 hrs, Figure 5A). HAP-ALEX signals at 16 hours and 24 hours overlapped very well with immunofluorescence. In this time frame we saw no evidence that HAP-ALEX caused turnover of Cp (Figure 5B), an activity attributed to some CpAMs.⁴⁶

We examined the dose dependence of HAP-ALEX on Cp distribution after treatment for 16 hr. Based on the EC_{50} of 0.7 μM , we anticipated little effect on Cp distribution at low HAP-ALEX concentrations. With 0.1 or 0.2 μM HAP-ALEX we observe a uniform CP distribution by immunofluorescence with no detectable concentration of HAP-ALEX (Figure 6A). At these low concentrations the Cp distribution of small puncta were indistinguishable from control experiments (e.g. Figure 4A). At HAP-ALEX concentrations 0.5 μM , Cp form much larger cytoplasmic structures (Figure 6A). At HAP-ALEX concentrations of 2 μM the puncta grew larger with stronger HAP-ALEX fluorescence (Figure 6A). A similar trend in puncta size and staining was also observed with longer durations of HAP-ALEX treatment (Figure 5, 24 hr panel). In the relatively short time frame of treatment (4 to 24 hrs), HAP-ALEX did not decrease the amount of Cp in cells as a function of CpAM concentration (Figure 6B) or time (Figure 5B).

***In vivo* HAP-ALEX also interacts with RNA filled and empty cores**

It is generally believed that maturation of the viral genome also affects core distribution and intracellular trafficking.^{50,51} To examine the effect of blocking genome maturation on the redistribution of Cp by HAP-ALEX, we expressed intracellular cores harboring the Y63F mutant polymerase. Although, these cores express and package the polymerase-pgRNA complex, reverse transcription is blocked.⁵²⁻⁵⁴ The presence of pgRNA in these Y63F

mutant cores was confirmed by quantitative RT-PCR; HAP-ALEX treated cores had only 67% pgRNA compared to DMSO treated Y63F cores (Supplementary Figure 3). It is to be noted that in our experiments the cells are treated for 16 hours with HAP-ALEX, 3 days post transfection, during which a substantial fraction of intracellular cores produced will package pgRNA. However, we do know from V124W mutation studies that this HAP pocket mutants only package 5% of pgRNA⁵⁵. Therefore, we speculate that any core produced during 16 hours of HAP-ALEX treatment may be significantly hampered in pgRNA packaging. Even in the absence of a HAP, in cultures and infections, a majority of cores are actually empty.⁵⁶ We observed no difference in the distribution of large cytoplasmic puncta induced by HAP-ALEX treatment (Figure 7A) in cells with and without functioning polymerase. Again, V124W mutant of the Y63F polymerase inactive clone showed no HAP-ALEX signal confirming specificity of HAP-ALEX binding (Figure 7B). To test if any other viral machinery was necessary for formation of large puncta, we tested expression of Cp by itself and found a similar effect (Figure 7C).

Conclusion

Antiviral compounds that target viral structural proteins are a new frontier. At their root, CpAMs change the physical chemistry of Cp assembly with profound biological effect. CpAMs, like HAP-ALEX, drive mis-assembly *in vitro* and *in vivo* lead to suppression of virus replication, relocalization of Cp to the cytoplasm, and formation of large puncta. Large puncta are suggestive of formation of the large aggregates seen *in vitro*. By using a fluorescent CpAM we can correlate effects on Cp localization with accumulation of the inducing small molecule. It is notable that relocalization and formation of large puncta occur temporally long before the drug accumulates (Figure 5A). The same Cp reorganization correlates well with the EC50 for antiviral activity. This result suggests that the CpAM at sub-stoichiometric concentrations, indicated by the relatively weak fluorescent labeling, can redirect Cp assembly and localization. Interestingly, HAP-ALEX sequesters Cp to cytoplasmic aggregates (Figure 5, 6). Over time, large puncta continue to grow and disproportionately more HAP-ALEX fluorescence associates with them (Figure 5). *In vitro*, we see a parallel effect. However, in biochemical experiments we need to approach one CpAM per Cp dimer to drive aberrant assembly *in vitro* (Figure 2), which implies that many of the large puncta in cells are not due to aberrant assembly but related to some other process. CpAM-induced assembly *in vivo* does not result in normal capsids as normal capsids do not form large puncta and are uniformly distributed cell-wide or in cytoplasm. In a normal infection a large fraction of Cp goes on to form empty capsids which can go on to acquire an envelope and be secreted along with mature virus.⁵⁷ Taken together, these data show that sub-stoichiometric concentrations of CpAM can induce Cp reorganization, fundamentally changing the morphology of an infected cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

HBV genomic clones used in the study, LJ144 (envelope protein deficient clone); LJ144-V124W (HAP insensitive mutant of LJ144), Y63F (polymerase mutant clone) and Y63F-V124W (HAP insensitive mutant of Y63F) were obtained from Daniel Loeb, U. Wisconsin, and pTruf-HBc (Cp clone) was a kind gift from Michael Nassal, U. Freiburg. We thank James Powers and the IU Bloomington Light Microscopy Imaging center, IU Bloomington Electron Microscopy Center and Angela Hansen at IU Mass Spectrometry Facility. We acknowledge the IU Electron Microscopy Center. We thank Shefah Qazi for her contribution to HAP-ALEX synthesis and Zhongchao Zhao for help with EM imaging.

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Figure 1. HAP-ALEX and its putative binding site.

(A) Capsid showing a single, highlighted core protein (Cp) dimer (yellow and pink) with hypothetical bound HAP-ALEX (green). (B) A model of HAP-ALEX in the HAP binding site is consistent with a recent structure with a TAMRA HAP derivative.⁴⁰ (C) The parent compound, HAP13 (1), and its ALEXA488 fluorescent derivative, HAP-ALEX (2).

Figure 2. Effect of HAP-ALEX on capsid assembly.

(A) HAP-ALEX has a dose dependent effect on the rate and extent of Cp149 assembly as observed by 90° light scattering (in arbitrary units). 10 μM Cp149 dimer was incubated with increasing concentrations of HAP-ALEX (black) or HAP13 (red) for 1 min at 25°C. The unlabeled lines are for 1.25 μM HAP-ALEX, 10 μM HAP13, and 0 μM HAP13. Assembly was initiated by adding an equal volume of 300 mM NaCl, 50 mM HEPES to attain a final concentration of 5 μM Cp149 dimer, 150 mM NaCl and 50 mM HEPES. The dashed blue line is the scattering of Cp149 capsid formed from 5 μM dimers in 150 mM NaCl; scattering above this intensity is indicative of aberrant assembly or large complexes. Each trace is an average of three independent experiments. (B) Negative stain electron micrograph of assembly products obtained with 15 μM HAP-ALEX; inset shows a normal capsid.

Figure 3. Binding of HAP-ALEX to Cp149 capsids.

Titration of 5 μM Cp149 with increasing concentrations of HAP-ALEX was observed by size exclusion chromatography. (A) Absorbance at 280 nm shows that HAP-ALEX increases the amount of capsids and non-capsid polymer at the expense of dimer. (B) Absorbance at 495 nm shows that HAP-ALEX co-elutes with the capsid/non-capsid polymer peak. No detectable HAP-ALEX co-eluted with the dimer peak. HAP-ALEX does adsorb to the column and elutes as a series of peaks at the end of the chromatograph. (C) Analysis of absorbance spectra shows that a maximum of one HAP-ALEX is bound per Cp dimer.

Figure 4. Detection of HBV intracellular cores by HAP-ALEX.

HuH7-H1 cells were transfected with an surface protein deficient (HBSAg⁻) clone of HBV. 3 days post-transfection cells were treated with DMSO or HAP-ALEX for 16 hours following which the cells were fixed and prepared for immunofluorescence (IF). (A) A control wild type transfection with a wild type Cp, treated with DMSO, and stained using a polyclonal anti-Cp (Dako). Note that the HAP-ALEX panel in this row is a blank. (B) A wild type transfection treated with HAP-ALEX and stained using polyclonal anti-Cp. (C) A wild type transfection treated with HAP-ALEX and stained using capsid specific monoclonal Mab3120. (D) Transfection with an HBSAg⁻ HBV clone with the HAP-resistant V124W mutant, treated with HAP-ALEX and stained using polyclonal anti-Cp. As predicted, the mutant failed to bind HAP-ALEX.

Figure 5. Detection of HBV intracellular cores by HAP-ALEX:Time course.

HuH7-H1 cells were transfected with the surface protein deficient genomic clone of HBV. 24 hours post-transfection cells were treated with HAP-ALEX for different times (4, 6, 8, 16, 24 hrs) after which the cells were fixed and incubated with anti-Cp (Dako) antibody for immunofluorescence (IF). Samples were visualized using a Leica SP8 confocal microscope. (A) With increased treatment time, HAP-ALEX labelling of Cp puncta increased. (B) Western blot analysis of the amount of intracellular CP during HAP-ALEX treatment for different durations of times (4, 6, 8, 16, 24hrs). HAP-ALEX did not cause any CP degradation during treatment.

Figure 6. Detection of HBV intracellular cores by HAP-ALEX: Effect of HAP-ALEX concentration.

(A) HuH7-H1 cells were transfected with the surface protein deficient genomic clone of HBV. 24 hours post-transfection, cells were treated with increasing concentrations of HAP-ALEX (0.1, 0.2, 0.5, 1, 2 μ M) for 16 hrs after which the cells were fixed and stained with anti-Cp (Dako) antibody (IF). Samples were visualized using a Leica SP8 confocal microscope. There is no detectable HAP-ALEX signal at concentrations lower than 0.5 μ M. At 0.5 μ M HAP-ALEX, Cp puncta become noticeably larger and show signs of ALEXA fluorescence. Size of puncta and the HAP-ALEX fluorescence intensity both increase with HAP-ALEX concentration. (B) Western blot analysis of the amount of intracellular CP during various HAP-ALEX treatment.

Figure 7. Detection of polymerase defective and empty HBV intracellular cores by HAP-ALEX. (A) HuH7-H1 cells were transfected with genomic clone of HBV that makes no envelope protein and encodes a Y63F mutant polymerase. These transfections will yield cores that contain pgRNA but are unable to synthesize rcDNA. 3 days post-transfection cells were treated with HAP-ALEX for 16 hours after which the cells were fixed and stained with anti Cp antibodies (Dako) for IF. (B) A parallel control experiment with expression of clone with defective Y63F polymerase and the V124W Cp mutant where Cp does not bind HAP-ALEX. (C) Expression of empty cores was accomplished by transfection of pTruf-HBc which encodes Cp but no other component of HBV. HAP-ALEX binds Cp and induces formation of large puncta under expression conditions that normally yield RNA-filled capsids (A) and empty capsids (C).