

BAY 41-4109 has multiple effects on Hepatitis B virus capsid assembly

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Here we report the effect of a heteroaryldihydropyrimidine (HAP) antiviral compound, BAY 41-4109, on Hepatitis B virus (HBV) capsid assembly and on preformed HBV capsids. The HBV capsid is an icosahedral complex of 120 capsid protein dimers. BAY41-4109 inhibits virus production *in vivo* by a mechanism that targets the viral capsid. We found that BAY 41-4109 was able to both accelerate and misdirect capsid assembly *in vitro*. As little as one HAP molecule for every five HBV dimers was sufficient to induce formation of non-capsid polymers. Unlike the related molecule HAP-1 (Stray *et al.*, *Proc. Natl. Acad. Sci. USA* 102:8138–43, 2005), no stable assembly intermediates were observed in assembly reactions with BAY 41-4109, indicating that accelerated assembly by BAY 41-4109 was still kinetically regulated by the nucleation rate. Preformed capsids were stabilized by BAY 41-4109, up to a ratio of one inhibitor molecule per two dimers. However, at BAY 41-4109:dimer ratios of 1:1 and greater, capsids were destabilized to yield very large non-capsid polymers. These data suggest the existence of two functionally distinguishable classes of drug-binding sites on HBV capsids. Occupation of the first class of site stabilizes capsid, while binding at the second class requires or induces structural changes that cannot be tolerated without destabilizing the capsid. Our data suggest that HAP compounds may inhibit virus replication by inducing assembly inappropriately and, when in excess, by misdirecting assembly decreasing the stability of normal capsids. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: Hepatitis B virus; virus assembly; antiviral drugs

Received 5 May 2006; revised 12 June 2006; accepted 11 July 2006

INTRODUCTION

Hepatitis B virus (HBV) is a major public health issue worldwide; an estimated 400 million people are chronically infected (Previsani and Lavanchy, 2002). In addition to sometimes-fatal acute infections, chronic HBV infections is the major risk factor for liver cancer (Hollinger, 1996).

Mature HBV virus particles consist of a 3.5 kb partially single-stranded circular genome, and a single copy of the viral reverse transcriptase encapsidated in an icosahedral protein capsid composed of 120 HBV capsid protein (Cp) dimers, host chaperones, and kinases may also be incorporated (Seeger and Mason, 2000). This 'core' (or hepatitis B core antigen, HBcAg) may be transported to the nucleus, or enveloped in membrane derived from the host endoplasmic reticulum, studded with HBV surface protein (or hepatitis B surface antigen, HBsAg). An effective and well-tolerated vaccine exists, but treatment options are currently limited to interferon,

which is expensive and frequently not well tolerated, and lamivudine and adefovir, nucleoside analogues which inhibit reverse transcriptase but have been shown to select for escape mutants. Recent drug screens have identified heteroaryldihydropyrimidines (HAPs) as a novel class of HBV inhibitors in tissue culture and animal models, effective at nanomolar concentrations (Weber *et al.*, 2002). Further research has shown that HAPs target Cp (Deres *et al.*, 2003), causing the formation of aberrant capsid at higher drug concentrations (Hacker *et al.*, 2003; Stray *et al.*, 2005).

We have recently shown that HAPs exert multiple effects at the level of capsid assembly (Stray *et al.*, 2005). At low concentrations, a representative HAP compound, HAP-1, caused an increase in the rate of capsid assembly *in vitro*, essentially acting as an allosteric activator. Assembly reactions with HAP-1 were observed to contain stable populations of assembly intermediates, predicted to occur when the rate of nucleation approaches the rate of elongation so that nucleation is no longer the rate-limiting step (Ceres and Zlotnick, 2002; Endres and Zlotnick, 2002; Stray *et al.*, 2004, 2005). At higher concentrations, HAP-1 induced the formation of aberrant particles, consistent with a preference for the formation of sheets of Cp hexamers in the presence of HAP-1, rather than the arrangement of Cp hexamers and pentamers found in normal viral capsids.

Here we examine the effect of another HAP compound, BAY 41-4109 (Figure 1), which has been tested in cell

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Abbreviations used: Cp, Capsid protein; EM, electron microscopy; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SEC, size-exclusion chromatography.

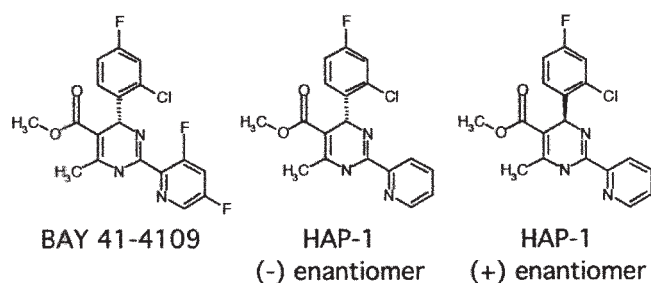


Figure 1. Structures of selected heteroaryldihydropyrimidines. Shown are BAY 41-4109 (Weber *et al.*, 2002) and (-) and (+) enantiomers of HAP-1 (Stray *et al.*, 2005). Note that BAY 41-4109 is pure (-) enantiomer (R enantiomer), whereas HAP-1 is a racemic mix.

culture and animal models (Weber *et al.*, 2002), and on HBV capsid assembly *in vitro*. Like HAP-1, BAY 41-4109 enhances the rate of assembly and induces aberrant particle formation at higher concentrations. Unlike HAP-1, BAY 41-4109 does not yield stable intermediates in *in vitro* assembly reactions, suggesting that, although assembly is faster, the rates of nucleation and elongation are both changed such that nucleation remains the rate-limiting step. We have defined two functionally distinguishable classes of site capable of binding HAP drugs, one of which can be occupied without disruption of capsid integrity, whereas occupancy of the second class of site destabilizes capsids.

MATERIALS AND METHODS

Protein expression and purification

Truncated HBV capsid protein (Cp149) was expressed and purified from *E. coli* as Cp149 dimers, as previously described (Zlotnick *et al.*, 1996, 2002). Protein was quantitated by absorbance at 280 nm ($\epsilon = 60900 \text{ M}^{-1} \text{ cm}^{-1}$ for Cp149). Where experiments required preformed capsids, these were assembled from concentrated dimer overnight at room temperature in 50 mM HEPES, 0.5 M NaCl, 5 mM DTT. Capsids were then purified by size exclusion over a superose 6 10/300 GL column using an Äkta chromatography system (both GE Healthcare, Piscataway, NJ). Capsids were stored in 50 mM HEPES, 0.5 M NaCl, 5 mM DTT at room temperature and used within 1 week.

Assembly inhibitors

The synthesis of both drugs has been described elsewhere (Weber *et al.*, 2002; Stray *et al.*, 2005, 2006). Note that HAP-1 is a racemic mixture of (-) and (+) (or R and S) enantiomers, whereas BAY 41-4109 is the pure (-) enantiomer (R enantiomer, see Figure 1) (Deres *et al.*, 2003). Master stocks of BAY 41-4109 (50 mM) and HAP-1 (10 mM) were maintained in DMSO and ethanol, respectively. Working stocks of both drugs were freshly prepared in 50 mM HEPES to eliminate apparent carrier effects of both solvents observed at low drug concentrations (data not shown). Both drugs precipitated from aqueous solution over time, so working stocks were not stored.

Light scattering (LS)

Assembly was monitored in real time by 90° LS in a SPEX Fluoromax-2 fluorometer (Jobin-Yvon SPEX, Edison, NJ) with a 0.3 cm path length cuvette (Hellma, Forest Hills, NY), as previously described (Zlotnick *et al.*, 2000; Stray *et al.*, 2005). Light scattering was measured at 400 nm with a 3 nm band pass, where absorbance of BAY 41-4109 and HAP-1 was negligible. Assembly was initiated by mixing 2x Cp149 plus 2x drug in 50 mM HEPES pH 7.5 with 50 mM HEPES pH 7.5-buffered 2x NaCl. Protein and NaCl solutions were pre-equilibrated to 40°C and assembly reactions were carried out at 40°C using a jacketed cuvette holder. The higher temperature enhances assembly, facilitating visualization of small kinetic differences.

Size exclusion chromatography (SEC)

Assembly reactions were examined by SEC on a Superose 6 10/30 column mounted on a Shimadzu HPLC system equipped with an auto injection module (Shimadzu, Columbia, MD). The column was equilibrated with 50 mM HEPES pH 7.5, 50 mM NaCl. For time course experiments, assembly was initiated robotically and allowed to proceed for the indicated time prior to automatic loading. Recovered protein was assigned either to the void (6.0–7.2 ml), capsid (7.2–8.3 ml), dimer (15–16.5 ml) or intermediate elution (8.3–15 ml), based on elution of reactions without drug, which contain only capsid and dimer.

Electron microscopy

Assembly reactions were prepared for EM as described (Zlotnick *et al.*, 2002). Samples were visualized on a Hitachi H-7600 transmission electron microscope and images collected using an AMT 2K × 2K CCD camera.

RESULTS

BAY 41-4109 enhances both rate and extent of HBV capsid assembly

We first examined the effect of BAY 41-4109 on assembly kinetics by monitoring real-time light scattering (Figure 2A). For 10 μM Cp149 assembly reactions containing 50 mM HEPES pH 7.5 and 150 mM NaCl incubated at 40°C, the rate of assembly was enhanced by as little as 0.2 μM BAY 41-4109, a ratio of 1 drug molecule per 50 dimers. Similar effects of BAY 41-4109 were seen for assembly reactions performed at room temperature using 5 μM Cp149 (data not shown). Because our studies are performed using much higher concentration than the 50 nM IC₅₀ and similar dissociation constant previously reported (Deres *et al.*, 2003), we expect BAY41-4109 to be bound stoichiometrically. In contrast, at least 0.5 μM HAP-1 was required for a similar effect ((Stray *et al.*, 2005), data not shown). Given that HAP-1 is a racemic mixture, and previous studies have shown that HAP binding is stereospecific, with only the (-) enantiomer capable of binding to

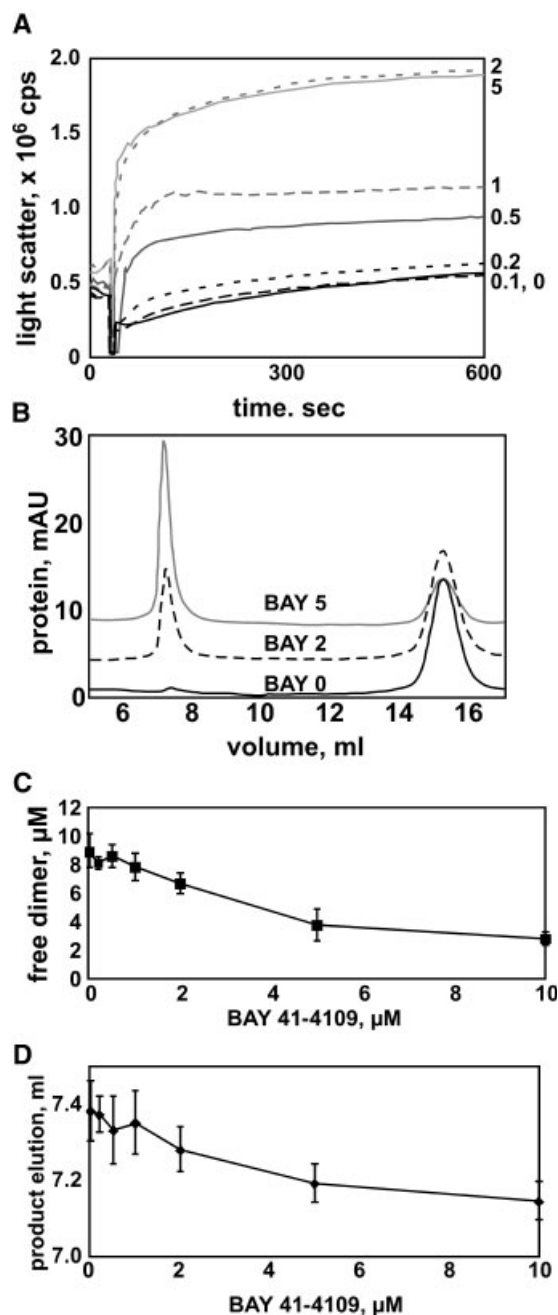


Figure 2. Effect of BAY 41-4109 on Cp assembly. Assembly of 10 μ M Cp dimer in 150 mM NaCl at 40°C was monitored by real-time 90° light scattering (A), or by size-exclusion chromatography after overnight incubation (B, C). The concentrations of BAY 41-4109 in assembly reactions (in μ M) are indicated to the left of the traces in panel A. Typical chromatograms are shown in panel B; chromatograms have been displaced vertically by 5 mAU for clarity. Capsid elutes at approximately 7.3 ml, dimer at approximately 15.5 ml and the void volume is at approximately 6.5 ml in this 20 ml Superose 6 column. The amounts of free dimer remaining after assembly were quantitated (C). Note that residual free dimer is inversely proportional to the amount of assembly product. Data are average $\pm$ 1 SD for three separate data sets, with each assembly reaction performed in triplicate in each data set. As the amount of BAY 41-4109 was added, the elution of the main product peak was progressively earlier in the chromatographic run (D). Only a single product peak was observed in each condition, suggesting a heterogeneous population of products of progressively larger average size at higher [BAY 41-4109]. Data are as described in panel C.

HBV core particles (Deres *et al.*, 2003), it is likely that BAY 41-4109 and the active form of HAP-1 (presumably 50% of the racemic HAP-1) would have almost identical effects on the rate of assembly.

We next examined the effect of BAY 41-4109 on the extent of assembly (Figure 2B,C). Because HAP-induced assembly can lead to capsids and large non-capsid polymers, which can be difficult to quantify, we are describing assembly reactions in terms of residual free dimer. Free dimer concentration is equal to the $K_{\text{Dapparent}}$ for capsids (Zlotnick, 1994) and the energetically near-equivalent critical concentration for open-ended polymers (Oosawa and Asakura, 1975). We found that BAY 41-4109 enhanced the extent of assembly (i.e. caused more dimer to be consumed) at lower concentrations than previously seen for HAP-1 (1 μ M BAY 41-4109 compared to 5 μ M for HAP-1, data not shown). In BAY 41-4109-containing assembly reactions, we detected only input dimer and capsid-sized (or larger) assembly products by size-exclusion chromatography (Figures 2B and 3). In contrast, we consistently observed assembly intermediates in assembly reactions containing HAP-1 (Stray *et al.*, 2005).

BAY 41-4109 misdirects assembly to the formation of aberrant products

Examination of the position at which the major product peak for BAY 41-4109 assembly reactions elutes shows that there is a smooth transition between capsid and 'void' product peaks as the amount of drug present in the reaction is increased (Fig. 2D). This is most likely due to a heterogeneous population of products. The shift in elution from capsid ($\sim$ 7.33 ml) towards the void suggests that the

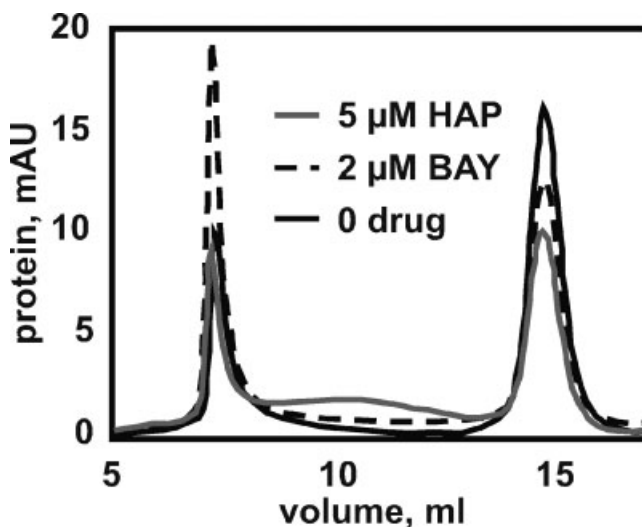


Figure 3. Comparison of the effect that BAY 41-4109 and HAP-1 have on assembly. Assembly of 5 μ M Cp in 0.5 M NaCl was examined by SEC over a superose 6 column after 16 h incubation in the presence of HAP-1 and BAY 41-4109, as indicated. No assembly intermediates were observed in the presence of BAY 41-4109, but are clearly evident in the presence of HAP-1. Because BAY 41-4109 is pure R enantiomer and HAP-1 is a racemic mixture, the two conditions represent approximately equivalent activities. The samples were run sequentially on the same column under identical conditions.

average size of the products is becoming larger. At higher concentrations ($\geq 5 \mu\text{M}$ BAY 41-4109), the majority of products are in the void. Increasing the concentration of BAY41-4109 results in less remaining free dimer, further demonstrating that BAY 41-4109, like HAP-1 acts to stabilize bound Cp149 relative to free dimer, as expected for an allosteric activator of assembly. When visualized by electron microscopy, the products of assembly with BAY 41-4109 are similar to those seen with HAP-1: balloon-shaped particles, spirals and small sheets are observed (Figure 4).

Effect of BAY 41-4109 on capsids correlates with stoichiometry

To better understand the mechanisms by which HAP drugs inhibit HBV replication, BAY 41-4109 was added to preformed capsids under a variety of conditions (Figure 5). We observed little effect on capsid stability when the ratio of BAY 41-4109 was less than one molecule drug per two molecules of dimer ($2 \mu\text{M}$ Cp plus $1 \mu\text{M}$ BAY 41-4109 or $4.8 \mu\text{M}$ Cp plus $2 \mu\text{M}$ BAY 41-4109, Figure 5 B,C). However, for drug concentrations around one drug molecule per dimer, capsids were destabilized and the resulting products resolve as two separate peaks, described as capsid and larger non-capsid 'void' oligomers (Figure 5A). At a stoichiometry of two drugs per dimer, all of the product is observed in the void. Examination of these reactions by electron microscopy yielded similar results: where capsids were incubated with BAY 41-4109 at ratios ≤ 1 molecule per Cp dimer, only capsid-like-structures were seen (Figure 6). Above this concentration, large sheet or tube-like structures were observed by electron microscopy, as seen previously

for HAP-1 (Stray *et al.*, 2005). Kinetically, no change in light scattering was observed until ≥ 1 drug molecule per dimer (results not shown). Similar effects were seen with HAP-1, at approximately double the HAP-1 concentration, consistent with HAP-1 being an approximately 50-50 mixture of two enantiomers, of which only one is biologically active.

Extent of capsid loss due to BAY 41-4109 depends on temperature

We have previously shown that capsid stability depends on temperature (Ceres and Zlotnick, 2002; Singh and Zlotnick, 2003; Stray *et al.*, 2005). BAY 41-4109 increased stability of assembled capsid and oligomer, as seen by the decrease in free dimer concentration (Figure 5B). Temperature did not affect the ≥ 1 drug molecule per dimer ratio at which assembled protein appeared in the void. However, at 4°C , the fraction of 'void' material seen was substantially greater at one drug molecule per dimer than seen at room temperature (Figure 5B,C). This may be due to decreased capsid stability at 4°C and/or a stronger effect of BAY41-4109 on re-arranging capsid protein. However, at all temperatures, we observed that lower concentration of BAY41-4109 stabilized capsid.

DISCUSSION

Though chemically and functionally similar, BAY41-4109 and HAP-1 show some distinct differences. HAPs have been shown to inhibit HBV virion production *in vivo* by targeting the capsid protein (Deres *et al.*, 2003). The mechanism by

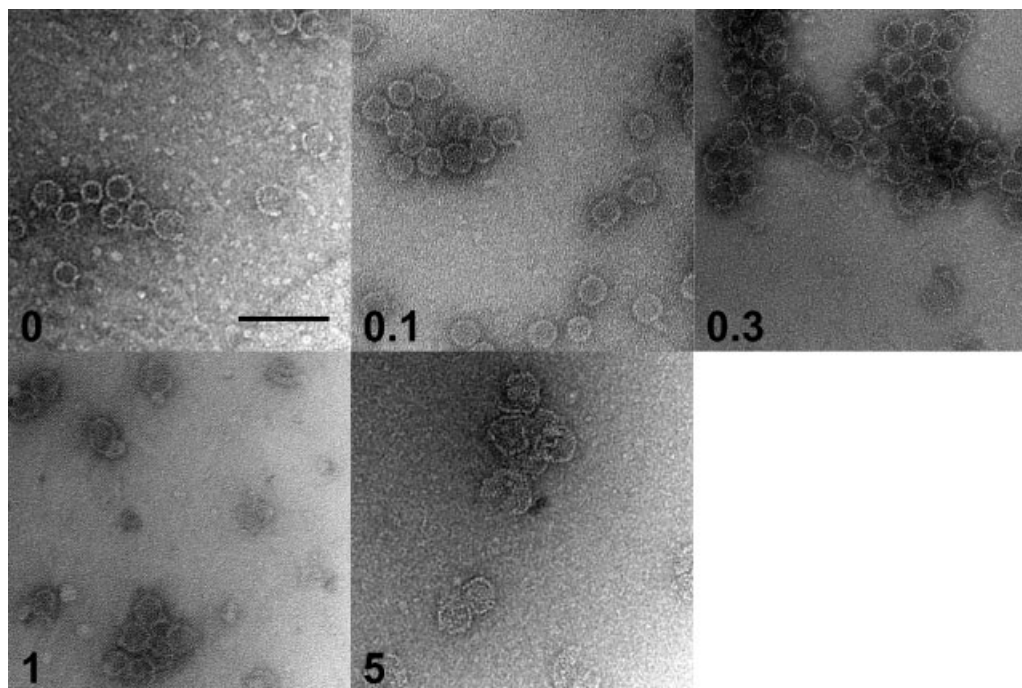


Figure 4. Products of assembly reactions examined by electron microscopy. Assembly reactions were performed at room temperature (23°C) with $5 \mu\text{M}$ Cp149 and 0.5 M NaCl. BAY 41-4109 concentrations (μM) were as noted on individual micrographs. At higher [BAY 41-4109] (indicated on micrograph), products were no longer uniformly spherical, but included sheets, cones and tubes of protein.

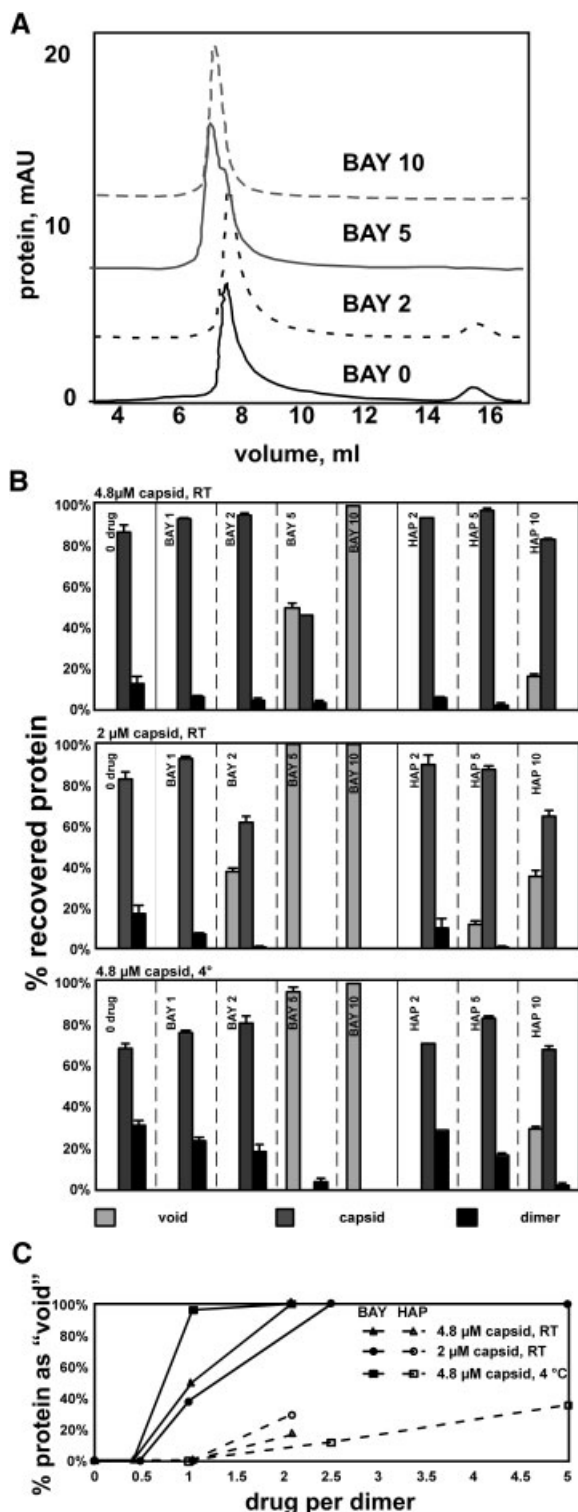


Figure 5. Effect of BAY 41-4109 on preassembled HBV capsids. Preassembled capsids were incubated at room temperature (23°C) in 150 mM NaCl with BAY 41-4109 overnight at the indicated concentrations (A, B); sample chromatograms have been displaced vertically by 4 mAU for clarity (A). Note that, unlike assembly reactions with free dimer (e.g. Figure 2B), there are distinct capsid and void peaks. The appearance of product in the void volume (rather than migrating as capsid) was measured as a function of [BAY 41-4109] (C). No void material was observed for drug concentrations representing a stoichiometry of ≤ 1 drug molecule per two dimers, but the presence of large non-capsid polymers is significant above this molar ratio.

which HAPs affect assembly of Cp149, the assembly domain of the core protein, is consistent with the loss of capsid integrity suggested by tissue culture results. In this study we have also uncovered another possible mechanism for HAP antiviral activity.

Both BAY 41-4109 and HAP-1 enhanced the rate of assembly of Cp at substoichiometric ratios of drug to Cp. Both drugs induced formation of aberrant structures at higher concentrations. Our studies with HAP-1 showed the presence of apparent assembly intermediates, which we interpreted as being on-pathway intermediates persisting due to the formation of a kinetic trap (Stray *et al.*, 2005). This is predicted to occur when the rate of nucleation approaches the rate of elongation, meaning that nucleation is no longer the rate-limiting step (Endres and Zlotnick, 2002; Stray *et al.*, 2004, 2005). Assembly with BAY 41-4109 showed no evidence of these stable intermediates, suggesting that this drug affects both nucleation and elongation reactions, whereas HAP-1 may preferentially speed up nucleation. Alternatively, the possibility must be considered that, of the two enantiomers of HAP-1 expected to be present, one may activate assembly while the other may have inhibitory effects.

Preformed capsids incubated with BAY 41-4109 tolerated a molar ratio of 1 drug molecule for every two dimers without any apparent change in size as determined by SEC and negative stained EM. At 1 BAY 41-4109 molecule per dimer (and greater), a fraction of the capsids dissociated and assembled material appeared in the 'void'. These data suggest that there are two functionally distinguishable classes of drug binding sites. One class of sites can be occupied without disrupting capsids. The structural changes required to fit the drug in the second class of site strain the capsids to such an extent that they are destabilized. The resulting drug-bound subunits and/or capsid fragments are consumed to form non-capsid polymers. At higher drug:dimer, the products we observed included sheets and tubes (largely hexameric arrays of Cp dimers (Stray *et al.*, 2005)). It should be noted that the proportion of capsids retained in the presence of drug is higher at higher temperature, where capsids are thermodynamically more stable (Ceres and Zlotnick, 2002; Singh and Zlotnick, 2003). It is very likely that the first class of sites is icosahedrally arrayed around the capsid: the first site stabilizes icosahedral capsids. The second functional class cannot be defined as clearly. It may also be icosahedrally arrayed, but have the effect of catastrophically destabilizing capsids when filled. However, it is more likely that the second class of sites is not symmetrically distributed as it is well tolerated in hexameric arrays of Cp but not capsids. This suggests that binding of HAP to this second class of sites can lead to the formation of defects in the capsid, from which dimers may dissociate, and support the formation of sheets. It should be noted that we cannot determine from the available data whether the HAP binding sites in sheets and tubes generated by assembly in the presence of HAP are identical to those found in the capsid. However, modelling studies showed that the dimer structures present in the HBV capsid crystal structure could be accommodated in hexagonal arrays with very few structural changes (Stray *et al.*, 2005), suggesting that capsids and HAP-induced assembly products are very similar up to the level of dimer quaternary structure.

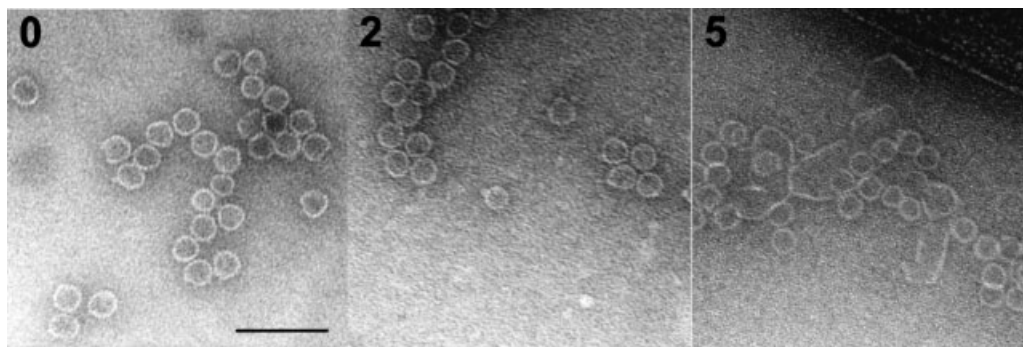


Figure 6. Reactions containing 5 μ M capsid and 150 mM NaCl incubated at room temperature (23°C) with BAY 41-4109 were examined by electron microscopy. BAY 41-4109 concentrations were as indicated on the micrographs. Note that these samples were from reactions included in Figure 4B. Only capsids were visualized at BAY 41-4109 ≤ 1 drug per two dimers; aberrant products are observed ≥ 1 drug per dimer.

At lower temperature, more capsids were lost: we observed a greater effect on capsid stability in the range 0.5–1 BAY 41-4109 per dimer at 4°C than at higher temperatures. We have shown that capsids are less stable at lower temperatures (Ceres and Zlotnick, 2002; Singh and Zlotnick, 2003). This arises because the assembly reaction is entropy driven, and thus assembly products are favoured at higher temperatures. The increased destabilization due to BAY 41-4109 at lower temperatures could simply reflect the lower stability of capsids at lower temperature, but another intriguing possibility is that drug binding sites become more accessible due to ‘breathing’, the transient dissociation of individual inter-dimer contacts, which is predicted to be greater at lower temperatures. HAPs may act to stabilize a metastable intermediate populated during the breathing process by binding the first class of site described above.

When a HAP is present during assembly, the capsid peak is not resolved from the non-capsid polymer peak. However, a separate non-capsid polymer peak is seen when a HAP is added to pre-existing capsids. The assembly products are essentially the same (compare Figures 4 and 6). We speculate that the average size difference (compare Figures 2 and 5) is a function of the availability of free dimer for nucleation (i.e. more dimer, as during *de novo* assembly, leads to more nuclei and smaller polymers).

The stoichiometry of binding of HAP drugs and its implications at the molecular level has not been clear until the availability of the X-ray crystal structure. Deres *et al.* (2003) originally reported a 1:1 binding of BAY 41-4109 to HBV Cp dimers, but their analysis was complicated by the fact that their system did not allow them to distinguish between drug bound to capsids, dimers or the non-capsid polymers we observe. In our studies, this value is consistent for non-capsid polymers, but not intact capsids. In a recent structure of HBV capsid co-crystallized with HAP-1 (Bourne *et al.*, in press), strong electron density consistent with putative drug binding was associated only with one of four classes of quasi-equivalent monomeric subunits, yielding a ratio of one HAP molecule per two dimers.

In addition to our previous proposal that HAP antagonizes HBV by causing the inappropriate assembly of capsid, we can add another possible mechanism: that HAPs bind capsids and may function by affecting capsid conformation and stability. For example, HAP binding may prevent some structural transition that may be necessary to activate reverse

transcriptase function or for correct budding (see also (Roseman *et al.*, 2005; Böttcher *et al.*, 2006)). HAP could also induce an aberrant transition to yield virus particles no longer competent for reverse transcription, budding and/or nuclear transport. This is consistent with the observation that there is a significant reduction of virus production in tissue culture when BAY 41-4109 concentrations were lower than those that affect the level of total Cp present in the cell (Deres *et al.*, 2003). Such a mechanism is analogous to that of antiviral compounds that inhibit picornavirus infection by preventing structural transitions (Smith *et al.*, 1986; Grant *et al.*, 1994; Lewis *et al.*, 1998; Tsang *et al.*, 2000). Thus, altering post-assembly transitions may be a very important aspect of the mechanism of HAPs.

The studies reported here demonstrate that two related small molecules have subtly different effects on HBV capsid assembly *in vitro*. These data suggest that HAPs antagonize HBV virion production by deregulating HBV capsid formation, and are also consistent with the possibility that the event targeted at the lowest drug concentrations is not capsid assembly *per se*, but a capsid structural transition, such as those required for production of mature virus or trafficking-competent cores. Capsid assembly remains further ground for deepening our understanding of the HBV lifecycle, as well as an important unexplored target for antiviral drugs.

Acknowledgements

We thank Dr Siegfried Goldmann, Department of Chemistry, Bayer Research Center, Wuppertal, Germany, for the kind gift BAY 41-4109. We thank Dr Christina Bourne for helpful discussions and critical reading of the manuscript. This work was supported by Research Grants from the National Institutes of Health (AI 67417-01) and the American Cancer Society (RSG-99339-04-MBC) to Adam Zlotnick.

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