

Inhibition of Hepatitis Delta Virus infection by Nucleic Acid Polymers - an *in vitro* analysis –



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Summary

Hepatitis delta virus (HDV) envelope proteins are derived from the hepatitis B virus (HBV). The HBV envelope proteins allows HDV to infect hepatocytes using HBV-specific cell surface receptor(s). The viral entry step is an attractive target for antiviral development because it concerns both HBV and HDV infections. In this study an *in vitro* HDV infection model was used to study the effects of various nucleic acid polymers (NAPs), on HDV infection.

The results demonstrate the following:

- DNA based NAPs (REP 2006, 2031 and REP 2055) can potently inhibit HDV infection.
- Inhibition of infection is sequence-independent but dependent on length and amphipathicity (phosphorothioation).
- The inhibitory effect occurs early in the HDV replication cycle, having no impact on the late HDV RNA replication step.
- Immobilized DNA-based NAPs efficiently bind HDV particles suggesting that these NAPs block viral attachment to cell surface heparan sulfate proteoglycans.
- DNA-based NAPs do not block HDV cell surface receptors since treatment of cells prior to inoculation does not prevent infection.
- NAPs are efficiently internalized in cells suggesting that they might also interfere with the HDV ribonucleoprotein trafficking to the nucleus or virion morphogenesis.
- **NAP entry inhibitory effects or interaction with HDV particles was blocked by the presence of 2'O-methyl modification of ribose, even in the presence of phosphorothioation.**

Nucleic Acid Polymers (NAPs)

Name	Sequence 5' - 3'	Length	Modifications			Chemistry
			PS	2'Ome (RNA)	5-MeC	
REP 2006	(N) ₄₀ (degenerate)	40	+			amphipathic (contains CpG)
REP 2107	(N) ₄₀ (degenerate)	40	+	+		amphipathic (contains CpG)
REP 2055	(AC) ₂₀	40	+			amphipathic
REP 2139	(AC) ₂₀	40	+	+	+	amphipathic
REP 2165	(AC) ₂₀	40	+	+	+	amphipathic (REP 2139 variant designed to degrade more rapidly)
REP 2172	(AC) ₂₀	40		+		non amphipathic (polyanionic) variant of REP 2055
REP 2147	(AC) ₂₀	40		+	+	non amphipathic (polyanionic) variant of REP 2139
REP 2149	(AC) ₃₀	60	+			REP 2055 size variants
REP 2150	(AC) ₁₅	30	+			
REP 2151	(AC) ₁₀	20	+			
REP 2152	(AC) ₅	10	+			
REP 2031	(C) ₄₀	40	+			
REP 2138	(C) ₄₀	40		+		amphipathic (neutralized at acidic pH, inactive <i>in vivo</i> against DHBV) non-amphipathic (polyanionic) variant of REP 2031

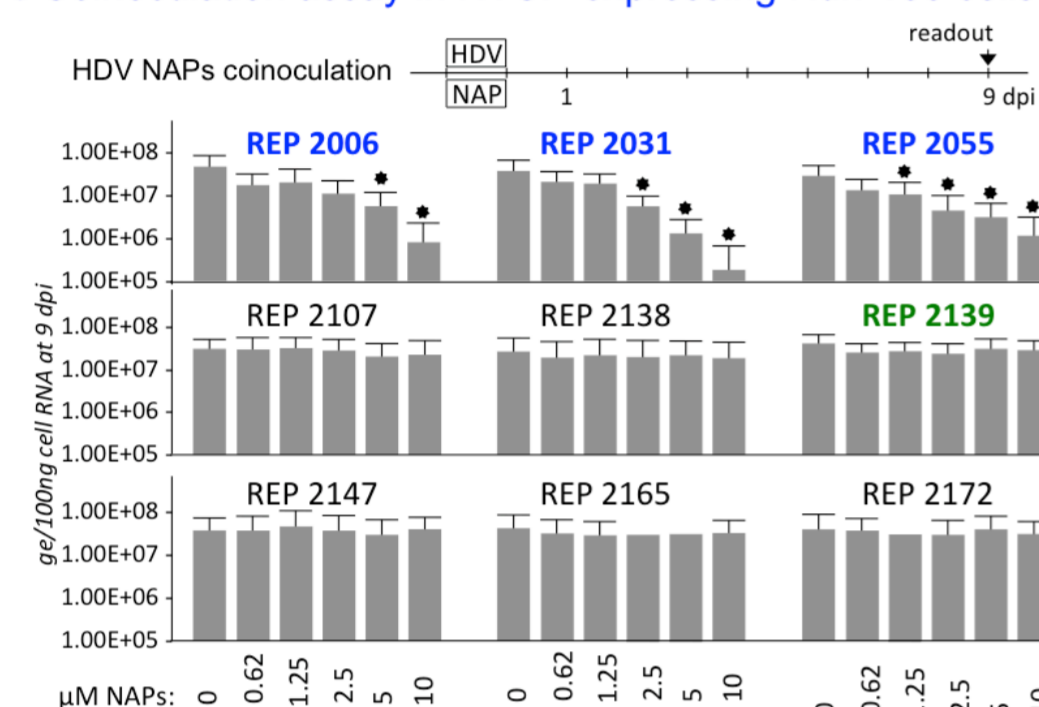
PS = phosphorothioation of phosphodiester linkage (increases amphipathicity)
2'Ome = O-linked methylation at 2' position in ribose (increased stability to nuclease attack and reduced TLR reactivity)
5-MeC = methylation of 5' position in cytidine base (reduced TLR reactivity)
* Positions 11, 21 and 31 have 2'OH ribose

Active against HDV *in vitro* (this study)

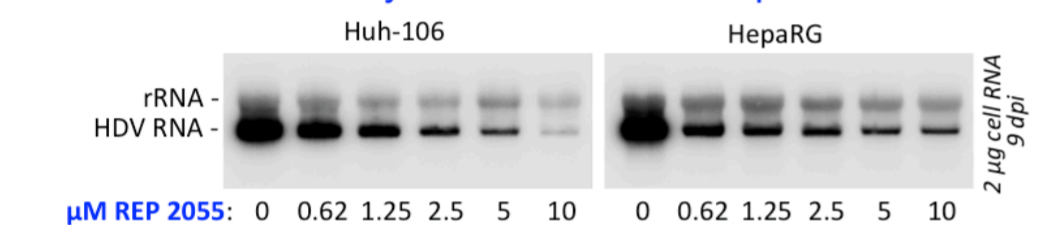
Active against HDV in human patients (REP 301 protocol NCT02233075) but not active in this study

NAPs inhibit HDV infection of human hepatocytes in culture

Coinoculation assay in NTCP-expressing Huh-106 cells

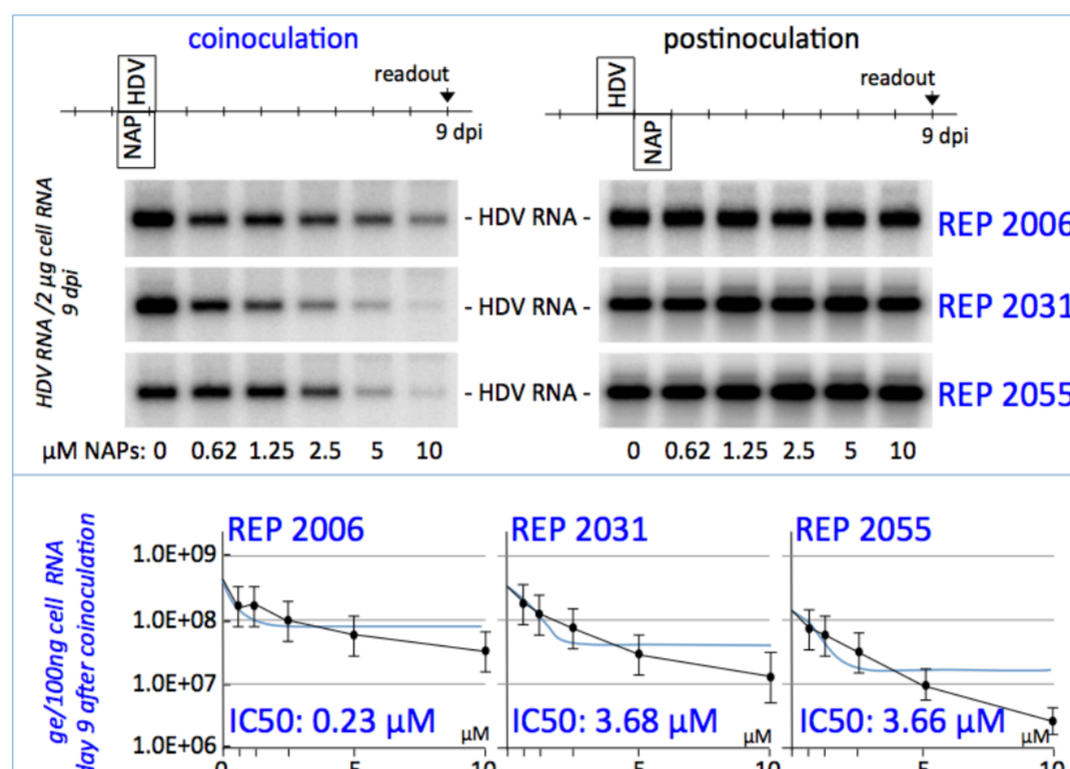


Coinoculation assay in Huh-106 and HepaRG cells



NTCP-expressing Huh-7 (Huh-106), or HepaRG cells, were exposed to HDV (500 ge/cell) in the absence (0) or presence of 1:2 dilutions of 10 μM of each NAP. Intracellular HDV RNA at 9 days postinoculation (dpi) was quantified by real-time qRT-PCR or Northern blot for measurement of infection. Values in the histograms are shown as means ± SD, expressed as ge per 100 ng of cellular RNA, in 3 independent experiments.

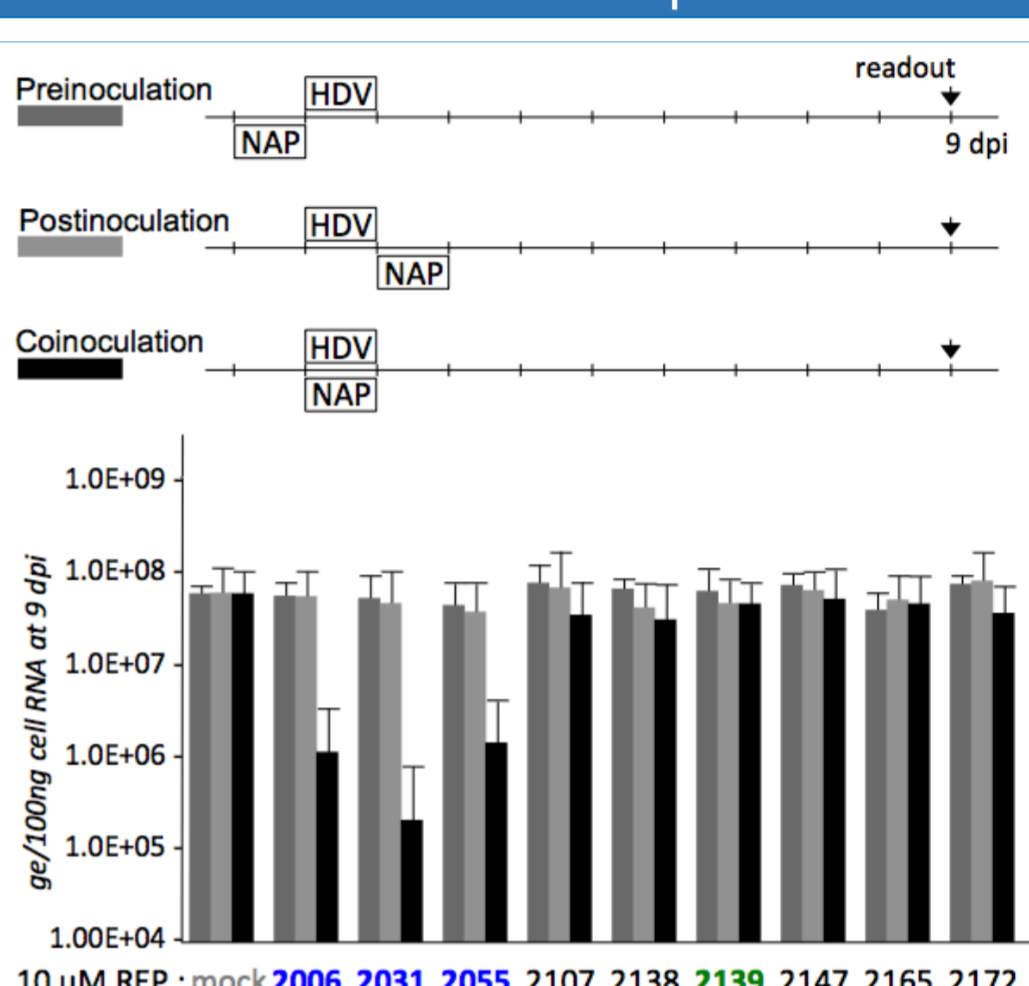
NAPs are active against HDV at submicromolar concentrations



Huh-106 cells were inoculated with HDV at 500 m.o.i., in the absence (0) or the presence of NAPs in: i) a coinoculation treatment, and ii) postinoculation treatment in which NAPs were added to cells for 24 h, after removal of the inoculum. At 9 dpi, intracellular HDV RNA was measured by real-time qRT-PCR. The values are shown as means ± SD, expressed as ge per 100 ng of cellular RNA, in 3 independent experiments. The IC50 was calculated using GraphPad Prism.

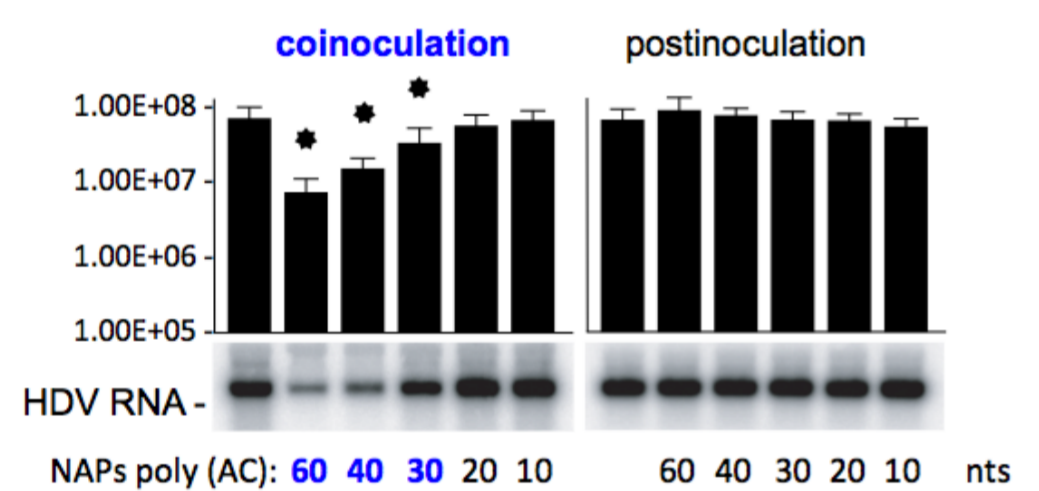
NAPs treatment of cells preinoculation does not prevent HDV infection

Treatment postinoculation does not inhibit HDV RNA replication



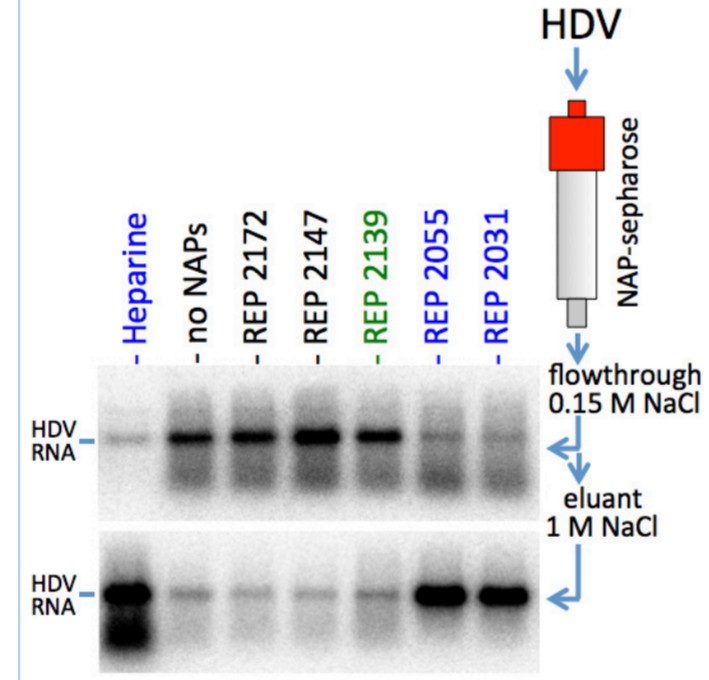
Huh-106 cells were inoculated with HDV at 500 m.o.i., in the absence (0) or the presence of 10 μM NAPs, under 3 conditions i) preinoculation: cells exposed to NAPs for 24 h prior to HDV, ii) postinoculation: treatment of cells with NAPs for 24 h, after removal of HDV. iii) coinoculation, in which cells were exposed to HDV and NAPs for 24 h. Intracellular HDV RNA at 9 dpi was quantified by real-time qRT-PCR. Values in the histograms are means ± SD, expressed as ge/100 ng of total cellular RNA, in 3 independent experiments.

NAPs inhibitory activity is size-dependent



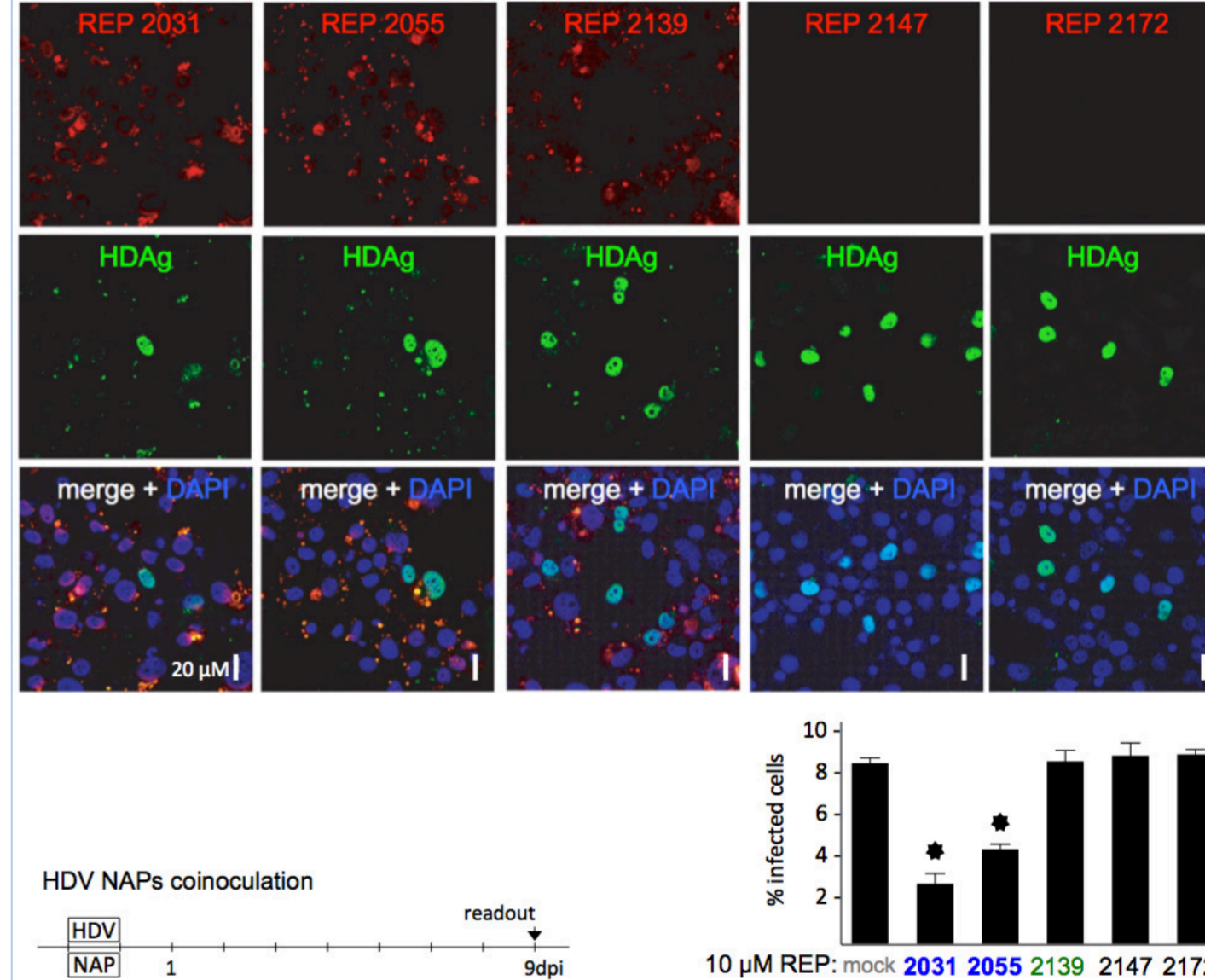
Huh-106 cells were inoculated with HDV at 500 m.o.i. in: i) a coinoculation setting with 2.5 μM of the indicated NAPs and HDV for 24 h and ii) postinoculation treatment in which NAPs were added for 24 h after HDV removal. At 9 dpi, intracellular HDV RNA was measured. Values in the histograms are shown as means ± SD, of HDV ge/μg of cellular RNA, in 3 independent experiments. Numbers at bottom indicate the NAPs size in nucleotides (nts).

Immobilized NAPs bind HDV



Biotinylated NAPs were bound to streptavidin-sepharose in a 400 μl volume column. After extensive washes in 150 mM NaCl solution, HDV-containing supernatants were loaded on the column. Flowthrough was recovered; columns were washed 5 x with 150 mM NaCl, and bound particles were eluted with 1 M NaCl solution. HDV RNA was measured by Northern blot analysis in flowthrough and eluate fractions.

NAPs present during HDV inoculation reduce % of infected cells and are internalized



Huh-106 cells were coinoculated with HDV and 10 μM of Cy3-tagged labeled REP-2031, -2055, -2139, -2147 and -2172. Infected cells were counted at 9 dpi using a human anti-HDAG antibody, and Alexa Fluor 488-conjugated anti-human IgGs (green). NAPs were detected at 9 dpi (red) and nuclei were labeled with DAPI (blue). Scale bars, 20 μm. The ratio of infected cells were plotted as % of HDV infected cells. Cytoplasmic green fluorescence in REP 2031, -2055 and -2139 treated cells was confirmed to be bleed-through from the red (NAP) channel and not HDAG derived (data not shown).

CONCLUSIONS

- The DNA-based NAPs, REP 2006, REP 2031 and REP 2055 inhibit HDV infection *in vitro* at submicromolar concentrations.
- Inhibition occurs specifically at the viral entry step by NAPs binding to the virus.
- Only NAPs that are amphipathic (phosphorothioated) are active against HDV infection, independent of the sequence of the NAP. The longer the NAP size (> 30 mer), the better the antiviral effect.
- **2'O-methyl modified NAPs, most notably the NAP active against HDV infection in patients (REP 2139), have no entry inhibitory effect.**
- The clinical activity of REP 2139 may thus be derived from an intracellular activity on HDV ribonucleoprotein trafficking and/or virions morphogenesis.
- Experiments are being designed to specifically address the effects of NAPs on HDV intracellular trafficking and morphogenesis.

REFERENCES

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