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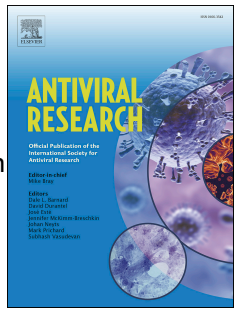

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Lipase inhibitor orlistat prevents hepatitis B virus infection by targeting an early step in the virus life cycle

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Abstract

Hepatitis B Virus (HBV) is a strictly hepatotropic pathogen which is very efficiently targeted to the liver and into its host cell, the hepatocyte. The sodium taurocholate co-transporting polypeptide (NTCP) has been identified as a key virus entry receptor, but the early steps in the virus life cycle are still only barely understood. Here, we investigated the effect of lipase inhibition and lipoprotein uptake on HBV infection using differentiated HepaRG cells and primary human hepatocytes. We found that an excess of triglyceride rich lipoprotein particles *in vitro* diminished HBV infection and a reduced hepatic virus uptake *in vivo* if apolipoprotein E is lacking indicating virus transport along with lipoproteins to target hepatocytes. Moreover, we showed that HBV infection of hepatocytes was inhibited by the broadly active lipase inhibitor orlistat, approved as a therapeutic agent which blocks neutral lipid hydrolysis activity. Orlistat treatment targets HBV infection at a post-entry step and inhibited HBV infection during virus inoculation strongly in a dose-dependent manner. In contrast, orlistat had no effect on HBV gene expression or replication or when added after HBV infection. Taken together, our data indicate that HBV connects to the hepatotropic lipoprotein metabolism and that inhibition of cellular hepatic lipase(s) may allow to target early steps of HBV infection.

Highlights

- excess of triglyceride rich lipoprotein particles competed with HBV infection
- the broadly active lipase inhibitor orlistat, approved as a therapeutic agent, targets HBV infection at a post-entry step

- the infection pathway of HBV requires hydrolysis of neutral lipids indicating a contribution of lipid or lipoprotein metabolism

Hepatitis B Virus (HBV) is a highly infectious pathogen specifically targeting hepatocytes and responsible for liver diseases such as cirrhosis and hepatocellular carcinoma. HBV undergoes a low affinity interaction with hepatocytes via heparan proteoglycans (Leistner et al., 2008; Schulze et al., 2007) before it binds NTCP, its bona fide receptor expressed on the basolateral, i.e. sinusoidal hepatocyte membrane (Yan et al., 2012). The physiological role of hepatocyte-specific NTCP is to mediate the uptake of bile acids from the blood circulation into hepatocytes.

The liver plays a key role in lipid metabolism. It takes up and oxidizes triglycerides (TG) to provide energy for its own purpose but also for other organs, and converts excess carbohydrates and proteins into fatty acids and TG, which are then exported and stored in adipose tissue. Furthermore, the liver synthesizes cholesterol, phospholipids and apolipoproteins (Apo). TG and cholesterol are hydrophobic neutral lipids that are both esterified either in the intestine or in hepatocytes before released into the bloodstream. They are transported in blood embedded in plasma lipoprotein particles containing Apo. Remaining cholesterol is excreted into bile or converted into bile acids to enable extraction and uptake of lipids from food. Lipoprotein particles enable fats and cholesterol to move within the water-based blood and are divided by their density relative to surrounding water into ultra-low density chylomicrons, very low (VLDL), intermediate (IDL), low (LDL) and high (HDL) density lipoproteins.

Ultra-low density chylomicrons transport lipids absorbed from the intestine to adipose, cardiac, and skeletal muscle tissue, where their triglyceride components are hydrolyzed by the activity of lipoprotein lipases, allowing the released free fatty acids to be absorbed by the tissues. When a large portion of the triacylglycerol core have been hydrolyzed, the chylomicron remnants become enriched with ApoE and ApoC2, the coenzyme for lipoprotein

lipase, and are taken up by hepatocytes. Within the hepatocytes remaining neutral lipids become hydrolyzed by cellular lipases (Ikonen, 2008).

VLDL are assembled within hepatocytes and released into the blood stream to transport "endogenous" TG, phospholipids and cholesterol forms. VLDL release TG and become IDL and LDL with ApoE determining their recycling to the liver. HDL particles remove cholesterol and fatty acids from cells and exchange neutral lipids with VLDL. ApoE is also present in a subfraction of HDL, HDL_E, where it serves as a major factor determining their rapid uptake into the liver (Richard and Pittman, 1993).

We have shown that Ezetimibe, a small molecule that was developed to bind and inhibit Niemann-Pick C1-like protein 1, a critical mediator of cholesterol absorption in the small intestine, efficiently inhibits HBV after uptake into hepatocytes (Lucifora, Esser, et al., 2013). In addition to blocking cholesterol transport, Ezetimibe strongly binds to NTCP (Dong et al., 2013) indicating structural similarities of the binding partners for both receptors, but also leaving the question open whether HBV might hijack lipid transport pathways for establishing itself in the hepatocyte.

We thus investigated whether lipoprotein uptake would affect HBV infection. In differentiated HepaRG cells (dHepaRG) (Gripon et al., 2002), an excess of triglyceride-rich lipoproteins (TRL) that encompass chylomicrons, chylomicron remnants and VLDL reduced HBV infection (**Figure 1A**) as shown by reduced levels of two typical markers of establishment of an HBV infection, the nuclear HBV covalently closed circular DNA (cccDNA) and the secreted HBe antigens (HBeAg). The competition between TRL and HBV for entry into hepatocytes suggests that the lipid uptake pathway supports HBV uptake into hepatocytes.

As mice are widely used as an *in vivo* model for liver TRL clearance and ApoE is a critical component of TRL (Dallinga-Thie et al., 2010), we next performed HBV uptake experiments in ApoE deficient compared to wild-type Bl6 mice. After i.v. injection of purified HBV that would be expected to associate with TRL if these are critical for its uptake into the liver, we observed a 35% reduction of viral particle uptake in the liver of *ApoE*$^{-/-}$ mice (**Figure 1B**) while the amount of HBV particles in their sera was approximately three times higher than in wild-type mice (**Figure 1B**). Additional reduction in viral liver uptake might be achieved when further TRL components involved in liver clearance like ApoV and lipoprotein lipase are addressed (Dallinga-Thie et al., 2010; Gonzales et al., 2013). Alternatively, the HBV stock used for infection may not completely dissociate from lipoproteins since virus purified from HepG2.2.15 cells was used. These data suggested that HBV may be taken up into hepatocytes together with lipoprotein-derived neutral lipids. The association of HBV with lipoproteins has already been emphasized by studies indicating that HBV binds to lipoprotein lipase via a PreS binding domain (Deng et al., 2007). An association of HBV to lipoproteins may also facilitate HBV targeting to NTCP (Yan et al., 2012) because bile acids bind lipoproteins and this association enhances bile acid binding to hepatocytes (Ceryak et al., 1993).

After uptake into hepatocytes, lipoprotein-derived neutral lipids become hydrolyzed before being further metabolized (Ikonen, 2008). We thus investigated if hydrolysis of neutral lipids might play a role in establishing HBV infection in hepatocytes. Treatment of dHepaRG cells with orlistat, an approved drug and broad inhibitor of mammalian neutral lipid lipases (Hadváry et al., 1991) interfered in a dose dependent fashion with the establishment of HBV infection if added prior to or during infection (**Figure 1C, 1D**), but not if added after infection (data not shown). IC50 and IC90 were respectively at 50 µM and 200 µM which is in line with

concentrations used for efficient block of intracellular lipase activity by orlistat *in vitro* (Mulder et al., 2004). A comparable inhibition of HBV infection by blocking the lipases was observed in primary human hepatocytes (**Figure 1E**). Importantly, when the HBV genome was transferred via an adenoviral vector circumventing the natural uptake pathway orlistat did neither affect HBV transcription, intracellular DNA replication or nuclear import of HBV capsids resulting in cccDNA establishment in this model (**Figure 1F**). To rule out orlistat inhibition of fatty acid synthesis (Kridel et al., 2004) we added C75, a specific inhibitor of fatty acid synthase, during HBV infection but observed no effect (data not shown). These results suggest that orlistat prevented HBV infection via inhibiting lipase activity that is needed during HBV particle uptake. Finally, we observed no toxicity of orlistat in the concentrations used in our experiments (**Figure 1G**).

In summary, our data indicate that HBV connects to the lipoprotein metabolism pathways to induce uptake into the liver and establish itself within the hepatocyte. Our data suggest that HBV might be taken-up into the liver along with lipoprotein-derived neutral lipids. This hypothesis is supported by genome-wide association studies. For lipoprotein binding of their receptor via ApoE the ApoE3 allelic variant generates a higher affine ligand than ApoE2 (Schneider et al., 1981). Interestingly, the ApoE3 allele is overrepresented among patients with HBV-related liver disease, and HBV-infected patients carrying the ApoE3 allele have a lower rate of HBsAg clearance (Ahn et al., 2012; Toniutto et al., 2010). These clinical observations maybe interpreted such that HBV infection is facilitated in humans carrying the ApoE3 allele and emphasize a role of lipoprotein targeting in HBV infection.

Moreover, we discovered that the broadly active lipase inhibitor orlistat, approved as a therapeutic agent, targets HBV infection at a post-uptake step. It does not affect transcription suggesting a role of neutral lipid hydrolysis activity in HBV fusion or

intracellular transport within hepatocytes. Using orlistat as a tagged probe for target identification might help to elucidate the still largely unknown steps in early HBV infection following immediate receptor binding. Although the plasma concentration of orlistat reported from *in vivo* studies (up to 16 µM) (Kridel et al., 2004) is lower than the IC50 we determined in cultured cells (approximately 50 µM) and thus effective antiviral activity was observed at a non-physiological range, orlistat might have the potential to support current antiviral therapy in a combination with established antivirals. Additionally, since the orlistat target enzyme in HBV infection might slightly differ from lipases reported to be specifically inhibited by orlistat (Hadváry et al., 1991), one could now investigate if a lead optimization may result in a higher target specificity with less systemic side effects.

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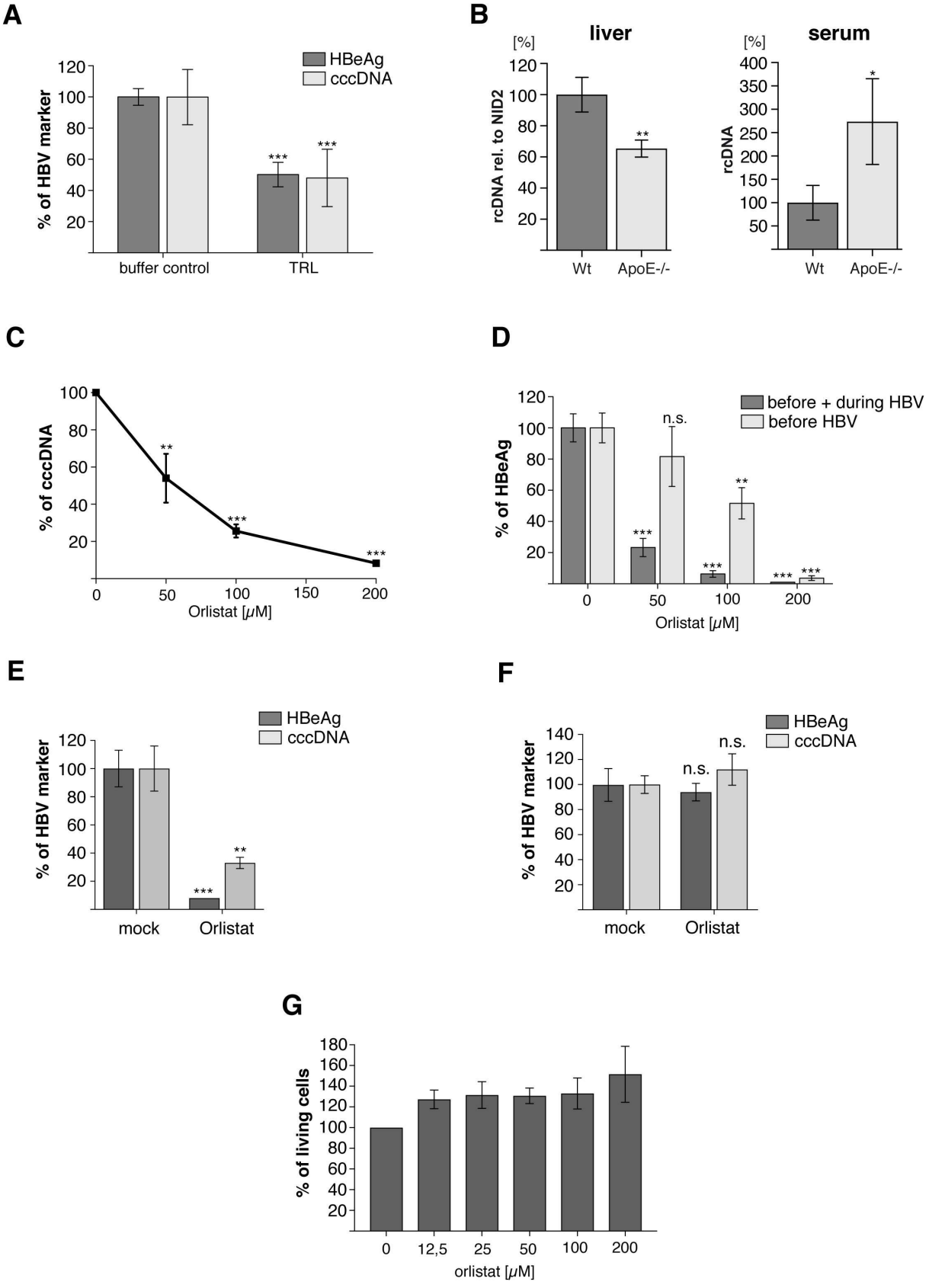
A
% of HBV marker
HBeAg
cccDNA
buffer control
TRL
B
liver
serum
[%]
rcDNA rel. to NID2
rcDNA
Wt
ApoE-/-
C
% of cccDNA
Orlistat [μM]
D
% of HBeAg
before + during HBV
before HBV
n.s.
Orlistat [μM]
E
% of HBV marker
HBeAg
cccDNA
mock
Orlistat
F
% of HBV marker
HBeAg
cccDNA
n.s.
mock
Orlistat
G
% of living cells
orlistat [μM]

Figure 1: Role of hepatic lipoprotein metabolism in early HBV infection stages and inhibitory effect of lipase inhibitor Orlistat

(A) Differentiated HepaRG (dHepaRG) cells were infected with HBV purified from HepG.2.2.15 cells by heparin columns at a multiplicity of infection of 200 DNA-containing virions / cell. 2h before and during infection, cells were incubated with 40 µg/ml TRL (0,96 – 1,006 g/ml; Acris Antibodies GmbH) or control buffer. 10 days post-infection, cell culture medium was analyzed for HBeAg by ELISA. Cells were lysed and cccDNA levels (normalized to *PrnP*) were analyzed by qPCR as described (Lucifora, Xia et al., 2014). **(B)** HBV purified by heparin column affinity chromatography, CsCl gradient and sucrose gradient ultracentrifugation was buffer exchanged to PBS and after addition of 40 µg/µl HSA to avoid unspecific uptake by phagocytic cells (Krone et al., 1990) 10^9 virions were injected into the tail vein of *ApoE*-/- mice (C57BL/6 background) (Piedrahita et al., 1992) or wild-type (Wt) C57BL/6 mice. After 30 min. mice were sacrificed and serum was harvested. Livers were lysed after perfusion with PBS and DNA was isolated. HBV-DNA genomes (rcDNA) were quantified by qPCR. **(C, D, G)** dHepaRG cells were treated with orlistat at indicated concentrations and **(E)** primary human hepatocytes with 100 µM orlistat, respectively, **(C, D, E)** before and during HBV infection or **(G)** for six days. **(G)** Cell viability was determined by MTT assays **(F)** dHepaRG cells were treated with 100 µM orlistat for 2h before transduction with a recombinant adenoviral vector containing a 1.3-fold HBV genome (AdHBV). Results are expressed in percentage of non-treated cells (mock) and were submitted to unpaired t-test statistical analyses; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.