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The retinoic acid receptor (RAR) α-specific agonist Am80 (tamibarotene) and other RAR agonists potently inhibit hepatitis B virus transcription from cccDNA

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ABSTRACT

Chronic infection with the human Hepatitis B virus (HBV) is a major global health problem. Hepatitis D virus (HDV) is a satellite of HBV that uses HBV envelope proteins for cell egress and entry. Using infection systems encoding the HBV/HDV receptor human sodium taurocholate co-transporting polypeptide (NTCP), we screened 1181 FDA-approved drugs applying markers for interference for HBV and HDV infection. As one primary hit we identified Acitretin, a retinoid, as an inhibitor of HBV replication and HDV release. Based on this, other retinoic acid receptor (RAR) agonists with different specificities were found to interfere with HBV replication, verifying that the retinoic acid receptor pathway regulates replication. Of the eight agonists investigated, RARα-specific agonist Am80 (tamibarotene) was most active. Am80 reduced secretion of HBeAg and HBsAg with IC_{50}s < 10 nM in differentiated HepaRG-NTCP cells. Similar effects were observed in primary human hepatocytes. In HepG2-NTCP cells, profound Am80-mediated inhibition required prolonged treatment of up to 35 days. Am80 treatment of cells with an established HBV cccDNA pool resulted in a reduction of secreted HBsAg and HBeAg, which correlated with reduced intracellular viral RNA levels, but not cccDNA copy numbers. The effect lasted for > 12 days after removal of the drug. HBV genotypes B, D, and E were equally inhibited. By contrast, Am80 did not affect HBV replication in transfected cells or HepG2.2.15 cells, which carry an integrated HBV genome. In conclusion, our results indicate a persistent inhibition of HBV transcription by Am80, which might be used for drug repositioning.

1. Introduction

Hepatitis B virus (HBV) infection causes chronic liver diseases (e.g. liver cirrhosis and hepatocellular carcinoma) and affects more than 240 million people worldwide. Despite the possibility of vaccination, the prevalence of HBV carriers remains high, as does the incidence of new infections, especially in sub-Saharan Africa and Asia (Stanaway et al., 2016; Schweitzer et al., 2015). There is no cure for chronic HBV infection. Approved treatment options (interferon-α, with important adverse effects and very moderate response rates, and nucleoside/nucleotide analogues, which rarely lead to HBsAg loss or seroconversion) are limited. Novel therapeutic strategies are therefore desired.

Abbreviations: all-trans retinoic acid, ATRA; cellular retinoic acid binding protein, CRABP; covalently closed circular DNA, cccDNA; Dulbecco's modified Eagle medium, DMEM; dimethyl sulfoxide, DMSO; farnesoid X receptor, FXR; Food and Drug Administration, FDA; hepatitis B core antigen, HBcAg; hepatitis B e antigen, HBeAg; hepatitis B surface antigen, HBsAg; hepatitis B virus, HBV; hepatitis D virus, HDV; immunofluorescence, IF; sodium taurocholate co-transporting polypeptide, NTCP; paraformaldehyde, PFA; peroxisome proliferator-activated receptor, PPAR; phosphate-buffered saline, PBS; polyethylene glycol, PEG; post infection, p.i; primary human hepatocytes, PHH; relaxed circular DNA, rcDNA; retinoic acid receptor, RAR; retinoic acid response element, RARE; retinoic X receptor, RXR; water soluble tetrazolium, WST-1

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HBV is a small, enveloped DNA virus with a highly-consolidated genome (Seeger and Mason, 2015). The HBV surface protein (HBsAg), consisting of large, medium and small variants; hepatitis B e antigen (HBeAg); and hepatitis B core antigen (HBcAg) are used as viral markers. Virus entry into hepatocytes initiates by specific binding of the viral large surface protein to human sodium taurocholate co-transporting polypeptide (NTCP) (Yan et al., 2012; Ni et al., 2014). In the nucleus, relaxed circular DNA (rcDNA) is "repaired" to covalently closed circular DNA (cccDNA). Pre- and subgenomic RNAs are transcribed from cccDNA by recruiting the cellular transcription system. Several host factors, among them nuclear receptors, and viral proteins regulate the transcription (André et al., 2011; Decorsière et al., 2016; Huan and Siddiqui, 1993; Quasdorff and Protzer, 2010; Radreau et al., 2016). Further, epigenetic modulation of associated histone proteins affects this process (Pollicino et al., 2006). After synthesis of structural proteins, newly-assembled nucleocapsids are enveloped by surface proteins (Blondot et al., 2016), or contribute to maintain the cccDNA pool in the nucleus (Nassal, 2015).

Hepatitis D virus (HDV), a satellite single-stranded RNA virusoid, uses HBV envelope proteins to package its ribonucleoprotein complex for spreading. Thus it enters hepatocytes via the same route as HBV and is sensitive to drugs affecting HBV surface proteins (Urban et al., 2014). Accordingly, HDV can be used as a pseudotyped reporter virus to screen for entry inhibitors and inhibitors affecting HBsAg.

We performed a screening of 1181 drugs approved by the United States Food and Drug Administration (FDA), to identify compounds with activity against HBV and/or HDV. We used HepG2 cells overexpressing NTCP, susceptible to both HBV and HDV, and HepG2 cells overexpressing NTCP together with the HBV envelope proteins, supporting HDV infection and allowing the secretion of progeny HDV. In combination, these cell lines allowed a differential screening of drugs with regard to inhibition of HBV/HDV entry, HDV replication and HDV release. Importantly, this screen can identify compounds inhibiting HDV directly by targeting HDV-specific replication, or indirectly by affecting HBV surface proteins.

The retinoid Acitretin was identified as an inhibitor of both HBV replication and the release of infectious HDV from cells. Retinoids bind to retinoic acid receptors (RAR), and/or retinoic X receptors (RXR), which are nuclear receptors and both exist in different isoforms (α/β/γ). RAR and RXR form heterodimers and bind to DNA regions called retinoic acid response elements (RAREs) (Giguere et al., 1987; Petkovich et al., 1987). Binding of retinoids to RAR affects the binding of transcription factors with various and even opposing effects (Poon and Chen, 2008). In hepatocytes, they play an important role in metabolism (Cunningham and Duester, 2015; Duester, 2008). Nuclear receptors, such as farnesoid X receptor (FXR) and RXR, are known to play a role in HBV transcription (André et al., 2011; Huan and Siddiqui, 1993; Quasdorff and Protzer, 2010; Radreau et al., 2016).

We further analyzed RAR agonists with respect to HBV infection and demonstrated interference with cccDNA-mediated transcription of RNAs predominantly in differentiated hepatic cell lines. We identified Am80, a very specific agonist of RARα, as the most active compound. Am80 is a 3rd-generation retinoid and approved in Japan for acute promyelocytic leukemia (Kanai et al., 2014; Shinagawa et al., 2014; Tobita et al., 1997). The high specific activity of Am80 (IC_{50} < 10 nM) makes it a promising candidate for *in vivo* preclinical and early efficacy studies in patients.

2. Materials and methods

2.1. Cells

HepaRG (Gripon et al., 2002), HepG2 and HuH7 cells stably expressing NTCP were described previously (Ni et al., 2014). Besides expressing NTCP, HepG2-derived HepNB2.7 cells (Lempp et al., 2019) contain an integrated HBV subgenome derived from the plasmid pT7HB2.7 (Sureau et al., 1994), which encodes the three HBV envelope proteins under authentic promoter control. Primary human hepatocytes (PHHs) were either bought from BioIVT (United Kingdom) or isolated from liver resections of patients undergoing partial hepatectomy as described previously (Vondran et al., 2007). The protocol was approved by the ethics commission of Hannover Medical School.

2.2. Drugs

An FDA-approved compound library was purchased from Prestwick Chemical. PA452 was bought from Tocris/R&D Systems (Cat. No. 5086). Acitretin (44707), 9-cis-retinoic acid (R4643), 13-cis-retinoic acid (R3255), all-trans-retinoic acid (R2625), Am80 (T3205), Tazarotene (T7080), Adapalene (A7486), Bexarotene (SML0282) and Ro41-5253 (SML0573) were purchased from Sigma-Aldrich.

2.3. HBV and HDV infection

PHH and HepaRG-NTCP celAls were inoculated overnight with HBV and/or HDV in medium containing 4% PEG and 1.5% DMSO, HepG2-NTCP, HepNB2.7 and Huh7-NTCP cells in medium containing 4% PEG and 2.5% DMSO (Ni and Urban, 2017). For details of virus production, see supplemental. HBeAg and HBsAg were quantified by Architect assay (Abbott), a commercial chemiluminescent microparticle immunoassay, by the Heidelberg University Clinic Analysis Center.

2.4. HBV transfection

Undifferentiated HepaRG-NTCP cells grown to about 70% confluence were used for transfection experiments. A plasmid containing the 1.3x overlength HBV genome (pT-HBV1.3) was transfected with TransIT-LT Transfection Reagent (Mirus) according to the manufacturer's instructions.

2.5. Assessment of cell viability

AlamarBlue cell proliferation and viability reagent (Bio-Rad) or water soluble tetrazolium (WST-1) cell proliferation reagent (Roche Diagnostics) were used according to the manufacturer's manuals.

2.6. Immunofluorescence (IF)

Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA). The following antibodies were used for staining: polyclonal rabbit anti-HBcAg (Dako; 1:1000 dilution) for HBV-infected cells; patients' sera positive for anti-HDV antigen (1:3000 dilution; gift from Raffaela Romeo, University of Milan) for HDV-infected cells; Alexa Flour-labelled goat anti-rabbit or goat anti-human (Invitrogen) secondary antibodies. Hoechst 33342 (Sanofi-Aventis) or DAPI (Sigma-Aldrich) were used for counter-staining of nuclei. Pictures were analyzed and quantified with ImageJ software.

2.7. Quantification of viral RNA, HBV total DNA and cccDNA

After harvesting cells and RNA extraction, cDNA was produced by reverse transcription. Pregenomic and total RNAs were then analyzed by quantitative PCR and normalization to GAPDH copy numbers. HBV total DNA was extracted from cells and supernatants with the Nucleospin tissue kit (Macherey-Nagel) according to the manufacturer's instructions. Quantitative PCR was performed as described (Qu et al., 2018). For cccDNA quantification, purified DNA was treated with T5 exonuclease to remove linear DNA and rcDNA, but not cccDNA (Qu et al., 2018). cccDNA copy numbers were assessed by quantitative PCR and normalized to beta globin (measured before T5 exonuclease treatment). Data were analyzed with the ΔΔCT method. For kits and primer sequences used, see supplemental.

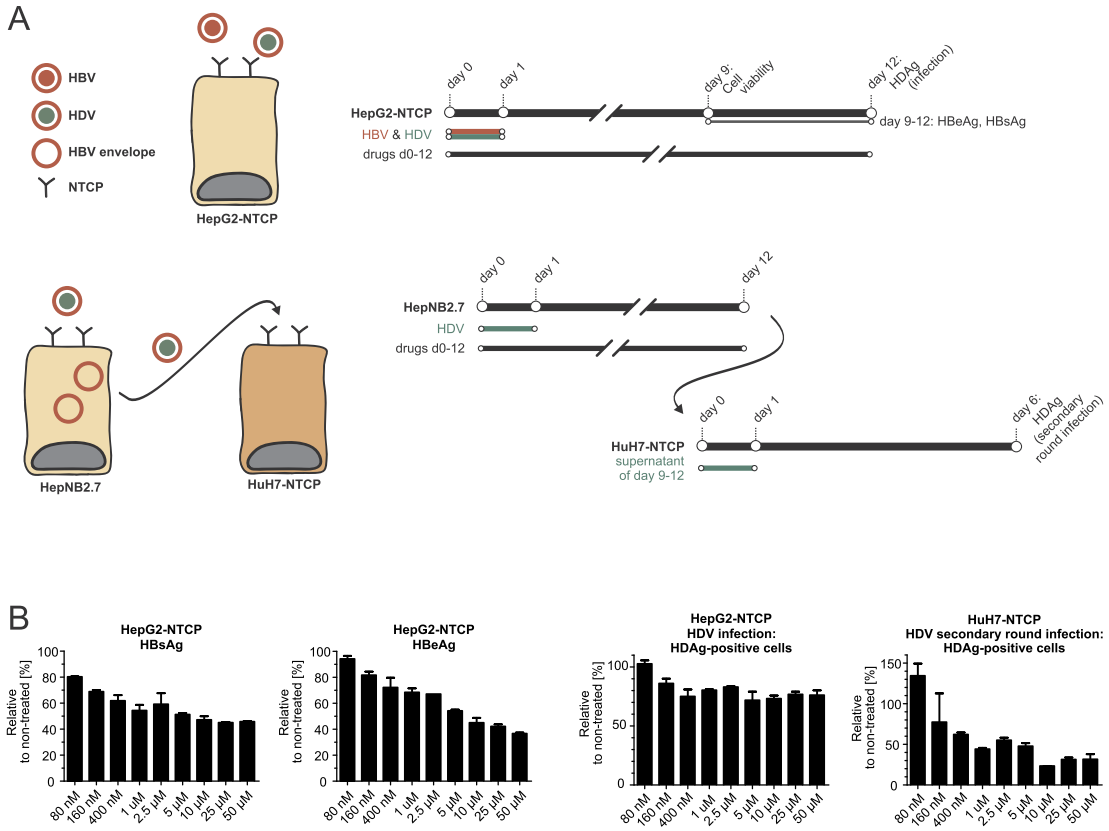

Fig. 1. Screening of the Prestwick FDA-approved drug library and validation of a primary hit. (A) Scheme of cell lines and time course of the initial screening approach. (Top) HepG2-NTCP cells were co-infected with HBV and HDV, the compounds to screen were applied during and after infection. Cell viability was assessed on day 9 p.i., HBV and HDV markers were analyzed on day 12 p.i. (Bottom) In parallel, HepNB2.7 cells were infected with HDV in the presence of the compounds. The cell supernatants were collected between days 9–12 and used to re-infect Huh7-NTCP cells, in which HDAg was assessed on day 6 p.i. (B) Results of the validation assay for Acitretin. Acitretin was supplied at increasing concentrations for an assessment similar to the one described above.

2.8. Assessment of RNA stability

PHH were infected with HBV and treated with 0 or 100 nM Am80 on days 3–4 p.i. On day 4, 10 µg/ml Actinomycin D (Sigma-Aldrich, A9415) was added. Intracellular RNA was harvested after 0, 3, 6, and 9 h and analyzed as mentioned above.

3. Results

3.1. A high-content screen of FDA-approved drugs identifies Acitretin as a specific inhibitor of HBV and HDV replication

HepG2-NTCP cells are highly susceptible to HBV and HDV infection in the presence of DMSO (Ni et al., 2014; Yan et al., 2012). HepNB2.7 cells support the complete HDV replication cycle (Lempp et al., 2019). These cell lines were used for a screening of 1181 FDA-approved compounds, depicted in Fig. 1A: HepG2-NTCP cells were co-infected with HBV/HDV and treated during and after virus inoculation for 12 days (upper panel). Viral markers were analyzed at day 12 p.i.: (i) secreted HBsAg, (ii) HBeAg; (iii) intracellular HBcAg by IF (see supplementary Fig. A1) and (iv) intracellular HDAg by IF. Cell viability was determined by the Alamarblue assay. In parallel, HepNB2.7 cells were mono-infected with HDV and treated during and after infection for 12 days (lower panel). The supernatants (containing progeny HDV) collected between days 9–12 p.i. were subsequently used for inoculation of HuH7-NTCP cells and HDAg quantification at day 6. With this, the effect of drugs on HDV entry, replication, as well as HDV release could be assessed by an optical read-out of HDAg-positive cells.

Three inhibitors with well-known modes of action were included as controls. The entry inhibitor Myrcludex B, as expected, blocked HBV/HDV primary infection and HDV secondary round infection. The prenylation inhibitor Lonafarnib interferes with HDV assembly and prevented HDV release in secondary round infection. The nucleotide analogue Tenofovir inhibits HBV DNA synthesis, but as expected did not prevent viral antigen production (supplementary Fig. A1, bottom). 24 hits from the screen were selected for validation (supplementary Fig. A1, top). Acitretin reduced HBsAg and HBeAg dose-dependently by > 54% at 50 µM (Fig. 1B). While HDAg was barely reduced following HDV primary infection, secondary round infection led to a dose-dependent reduction of infected cells up to 77% at 10 µM, while cell viability was not reduced. This indicates that Acitretin inhibits HBV and may indirectly interfere with HDV reinfection by affecting the release of progeny HDV.

3.2. RAR agonists affect HBV replication after establishment of cccDNA

To differentiate whether Acitretin acts at early or later infection steps, HepaRG-NTCP cells were treated continuously during and after HBV infection, or with a delay of 3 days p.i. Both settings reduced HBsAg and HBeAg secretion, with only moderately lower IC_{50}s when the drug was applied without delay (Fig. 2A), hinting at a major effect late in the replication cycle.

We investigated several retinoic receptor agonists with different specificities for RARα/β/γ and RXR (Fig. 2B–C; supplementary Fig. A2). All RAR agonists inhibited HBsAg secretion, with profound differences in their IC_{50}s (1.9 nM for Am80 - 2.2 µM for 13-cis RA). The RXR-

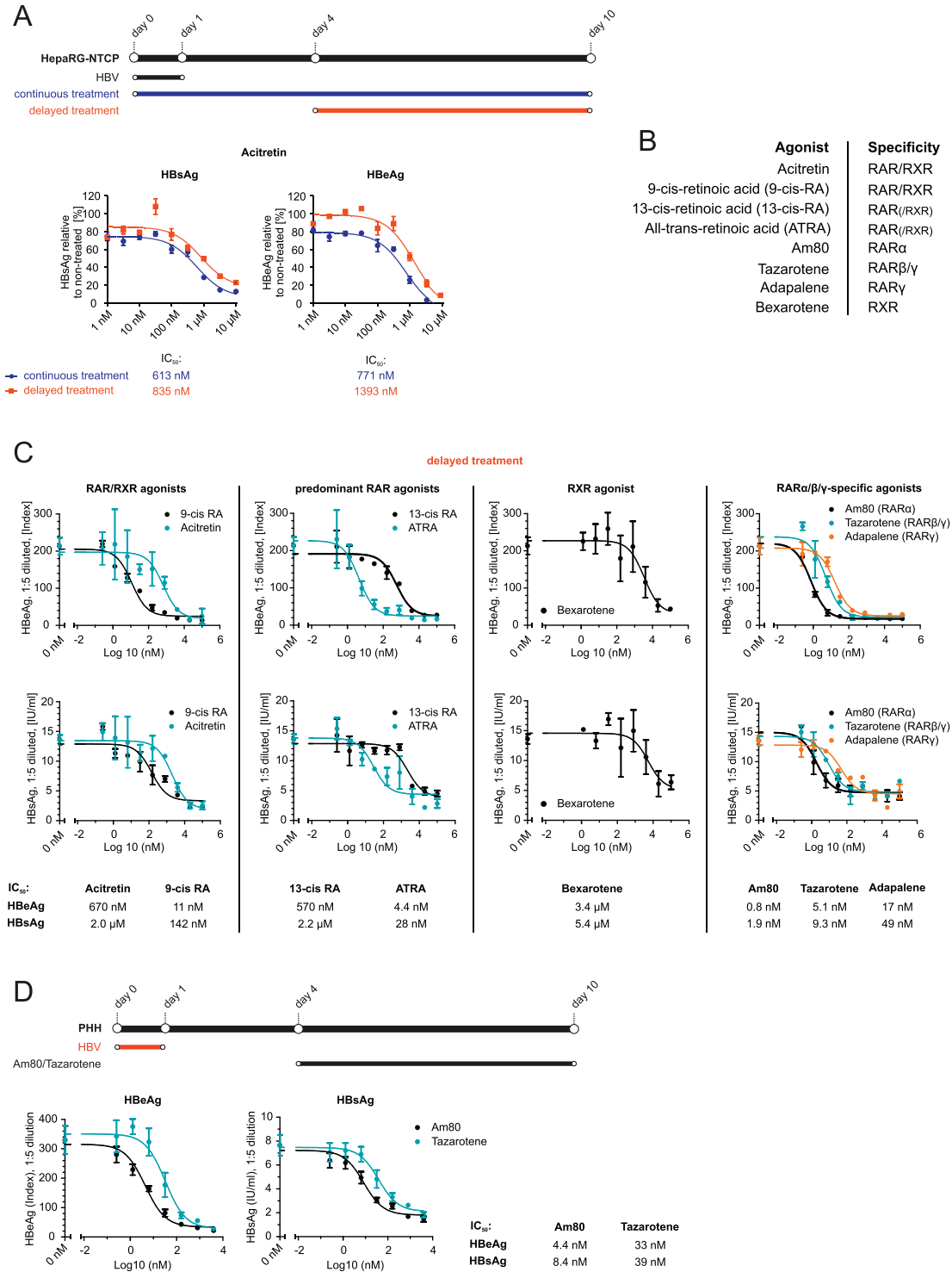

Fig. 2. Comparison of the activities of Acitretin and other RAR/RXR agonists on HBV infection in differentiated HepaRG-NTCP cells and PHH. (A) (Top) In HepaRG-NTCP cells, Acitretin was applied during and after HBV infection (continuous treatment) or administered starting 4 days p.i. (delayed treatment) (Bottom). Dose-response activity of Acitretin on the secretion of HBsAg and HBeAg in a continuous and delayed treatment setting and determination of the respective IC_{50} values. (B) List of the retinoic receptor agonists investigated in this study and their specificity for RAR/RXR. (C) Antiviral activity (measured as secreted HBeAg and HBsAg) of different RAR/RXR agonists during delayed treatment of differentiated HepaRG-NTCP cells. (D) Treatment scheme and dose-response curves of the two most potent RAR agonists, Am80 and Tazarotene, in PHH, with IC_{50}s for HBeAg and HBsAg. Note that HBsAg cannot be reduced by more than 80%.

specific agonist Bexarotene was least active (IC_{50} 5.4 µM). IC_{50}s for HBsAg were generally lower than those for HBeAg. We further examined the effect of the retinoic receptor antagonists Ro41-5253 and PA452 (supplementary Fig. A3). Even when applied continuously, the RARα antagonist Ro41-5253 did not inhibit secretion of HBsAg and HBeAg. The RXR antagonist PA452 showed a very limited decrease of viral marker secretion (45% HBeAg and 20% HBsAg reduction at 10 µM).

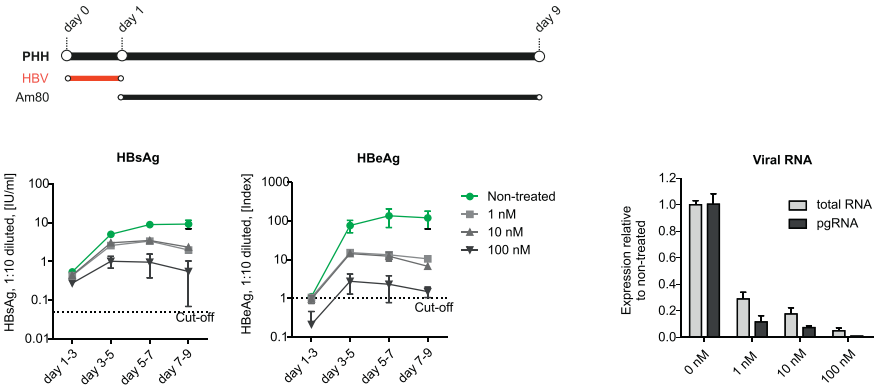

Fig. 3. Time-dependent increase of the Am80 inhibitory activity in PHH. Am80 was added immediately after HBV infection of PHH. Medium including the drug was exchanged and HBsAg and HBeAg were measured every two days p.i.; HBV total RNA and pregenomic RNA were determined by RT-qPCR on day 9 p.i.

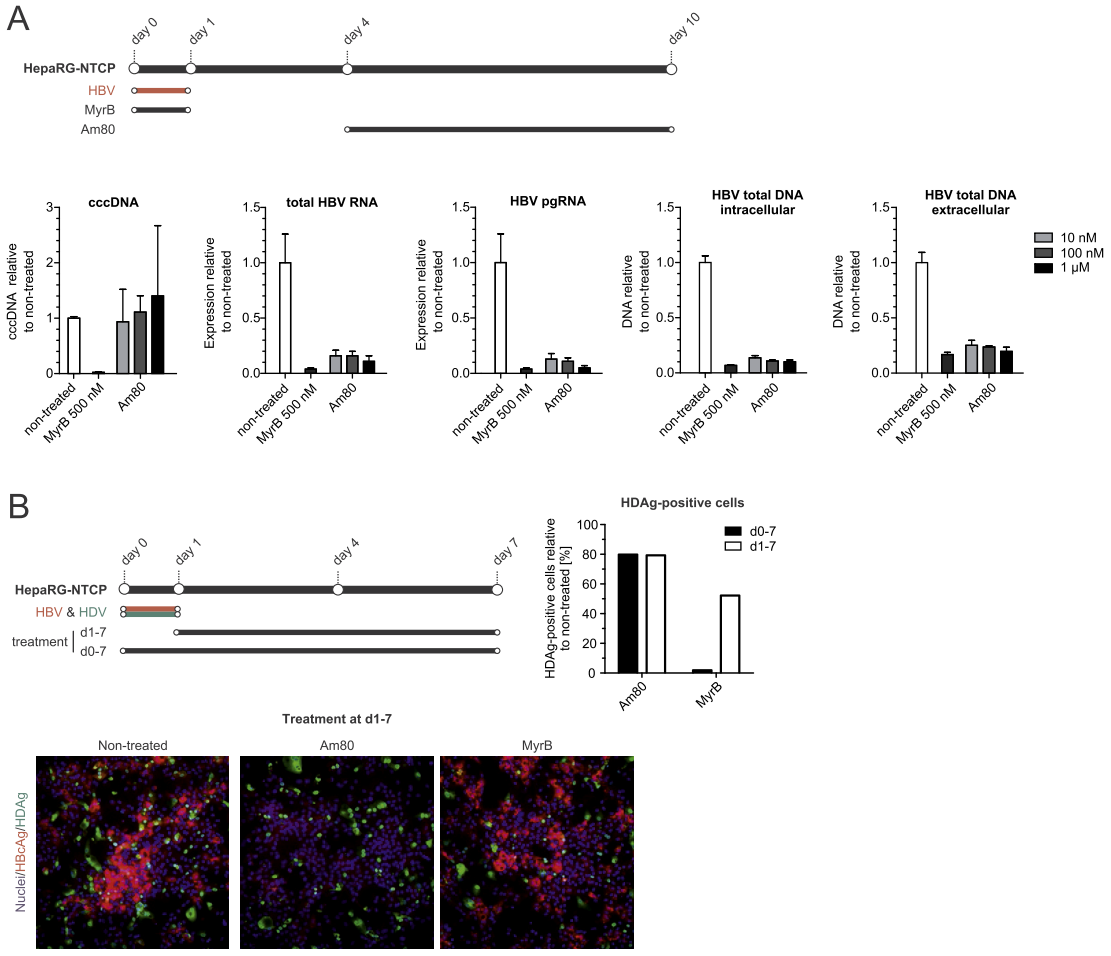

Fig. 4. Am80 inhibits HBsAg and HBeAg secretion and reduces viral transcription from cccDNA. (A) HBV infection of HepaRG-NTCP cells. The entry inhibitor Myrcludex B was applied during virus inoculation as a control for specific infection, Am80 was applied in a delayed manner starting on day 4 p.i. HBV cccDNA at day 10 p.i. was determined following T5 exonuclease digestion; HBV total and pregenomic RNA were determined in parallel by RT-qPCR. Intracellular and extracellular levels of HBV total DNA were as well quantified by PCR. (B) Selective activity of Am80 on HBV. HepaRG-NTCP cells were co-infected with HBV and HDV. Am80 or Myrcludex B was applied during or immediately after infection, with a medium change on day 4. IF read-out on day 7 p.i.

3.3. Am80 efficiently decreases HBV antigen secretion and HBV RNA transcription, but does not affect cccDNA copy numbers

Of all tested RAR agonists, Am80 was most potent. We determined IC_{50}s of 0.8 nM (HBeAg), respectively 1.9 nM (HBsAg), when HepaRG-NTCP cells were treated for 6 days (Fig. 2C). The RARβ/γ agonist Tazarotene was recently described to inhibit HBV (Li et al., 2018), at IC_{50}s consistent with our results. We directly compared both drugs in PHH (Fig. 2D). As in HepaRG-NTCP cells, Am80 was around 5-fold more potent than Tazarotene. Toxicity of Am80 was only observed at concentrations > 1 µM (supplementary Fig. A4). In HBV infection of PHH, Am80 also showed a time-dependent increase in its activity on HBV infection markers (Fig. 3). To next investigate whether the reduction of HBsAg and HBeAg reflects a reduction of HBV transcription, we determined HBV RNAs by quantitative PCR. HBV total and pregenomic RNA were decreased by 95%, respectively 99% in PHH (Fig. 3) and by

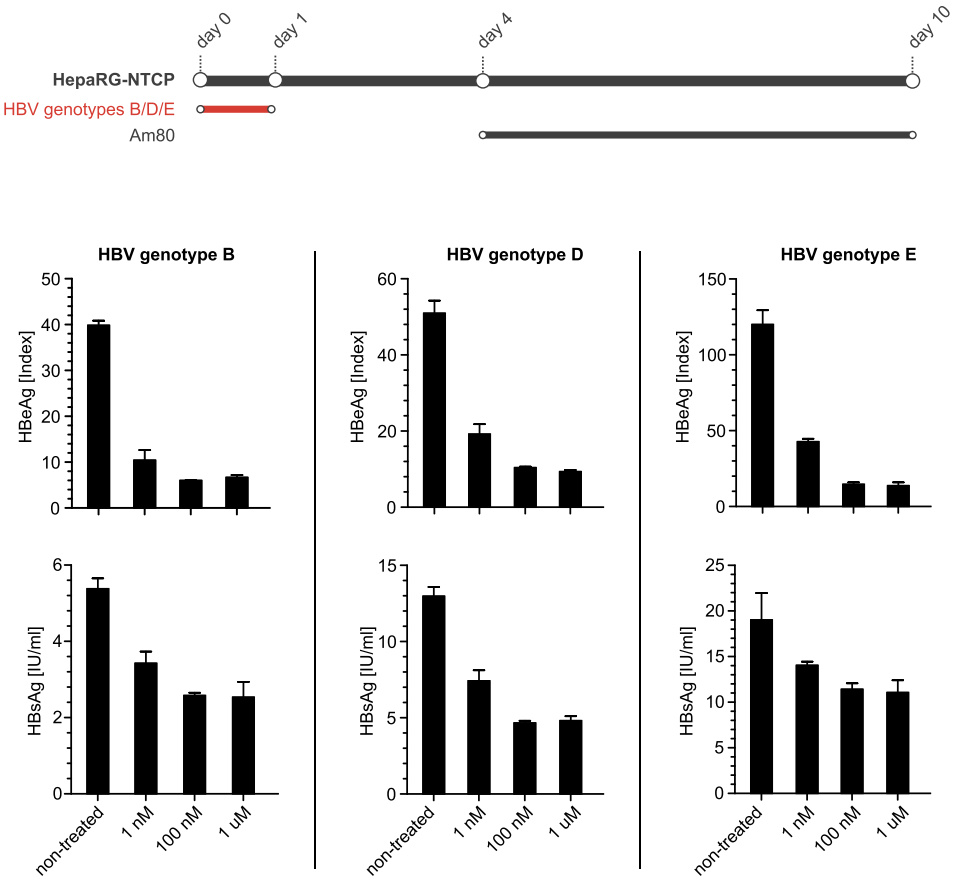

Fig. 5. Am80 inhibits different HBV genotypes. HepaRG-NTCP cells were infected with HBV genotypes B, D, and E, and treated with Am80 in a delayed manner according to the scheme. Secreted HBeAg and HBsAg were measured between days 7 and 10 p.i.

84%, respectively 89% in HepaRG-NTCP cells upon Am80 treatment. An RNA stability assay showed no difference in HBV RNA decrease of Am80-treated and -untreated PHH, confirming that the reduction of HBV RNA was not due to decreased stability (supplementary Fig. A5). Intracellular and secreted HBV total DNA levels, reflecting replication intermediates, were likewise decreased. Intracellular HBcAg was also distinctly reduced. By contrast, cccDNA levels, quantified after T5 exonuclease-mediated removal of rcDNA-containing replicative intermediates (Qu et al., 2018), were not affected (Fig. 4A).

The strong activity of Am80 in a delayed treatment setting indicates that its major effect does not affect NTCP-mediated entry. We further tested Am80 on HDV infection (as both viruses use the same entry route). HepaRG-NTCP cells were co-infected with HBV/HDV in the presence of Am80 during and after or only after infection (Fig. 4B). While a profound interference with HBV replication could be verified by HBcAg staining, HDV infection was only marginally reduced (by 20%), as judged by the numbers of HDAg-positive cells.

We also compared the efficacy of Am80 against infection with different HBV genotypes. When treated in a delayed setting (days 4–10 p.i.), HBV genotypes B, D, and E were equally inhibited (Fig. 5).

3.4. Am80 does not affect HBV replication initiated from transfected or integrated HBV DNA

Next, we examined the effect of Am80 on cells infected with HBV compared to those transfected with a plasmid encoding the HBV genome (Fig. 6A). In contrast to Am80 in infected HepaRG-NTCP cells without prior differentiation, Am80 in transfected cells did not reduce secreted viral markers.

In addition, we tested the activity of Am80 in HepG2.2.15 cells, which carry an integrated HBV genome and constitutively secrete HBeAg and HBsAg independent of cccDNA. When treated for 6 days with Am80, there were no significant effects on both markers (Fig. 6B). Since HBV replication driven by transfected plasmid or integrated genome, unlike infection, does not rely on cccDNA formation, Am80 most likely regulates transcription specifically from cccDNA.

3.5. Long-term infection reveals a time-dependent and sustained effect with marginal rebound at least 12 days after drug withdrawal

We tested a possible enduring effect of Am80 in HepaRG-NTCP and HepG2-NTCP cells, as these cell lines can be cultivated for several weeks (Fig. 7). Starting from day 7 p.i, HepaRG-NTCP cells were treated with Am80 for 3 weeks (Fig. 7A). One week after start of treatment, both HBsAg and HBeAg were substantially reduced as shown before (see Fig. 2C). Remarkably, continuation of treatment to 3 weeks nearly abolished antigen secretion. This indicates a progressively accumulating activity.

We performed a similar experiment with the hepatoma-derived cell line HepG2-NTCP. After treatment with Am80 on days 7–14 p.i., HBeAg and HBsAg secretion was less inhibited, compared to HepaRG-NTCP cells and PHH. However, when treatment was extended to 3 or 5 weeks, HBV inhibition was considerably increased (Fig. 7B). This shows that at all three cell lines, although having pronounced differences in their kinetics, are sensitive to interference with the RARα-mediated signalling pathway.

To assess a possible virus relapse after treatment, we treated HBV-infected HepaRG-NTCP cells directly after infection with 100 nM Am80 for 6 days and cultivated the cells for 12 further days after drug removal (Fig. 7C). Remarkably, the effect of Am80 lasted and viral markers remained decreased with only a marginal rebound at later time points, indicating that transcriptional repression was persistent for at least 12 days.

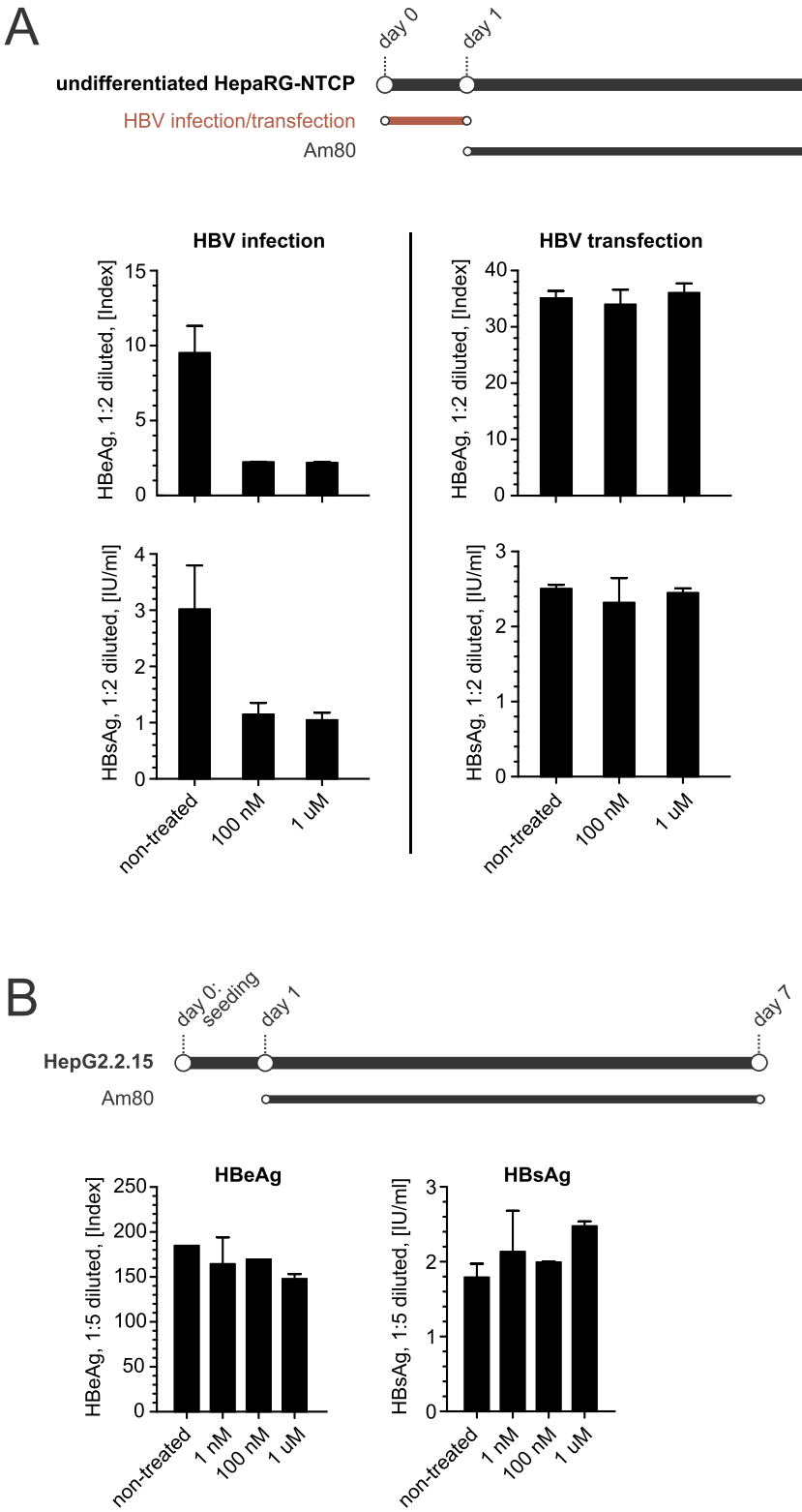

Fig. 6. Am80 has no effect on HBV transcription in transfected hepatocytes and in a cell line with integrated HBV genome. (A) Undifferentiated HepaRG-NTCP cells were either infected with HBV, or transiently transfected with a plasmid encoding a 1.3-mer more-than-genome-length HBV genome, and subsequently treated with Am80 (1 nM, 100 nM and 1 μM) for 6 days. HBeAg and HBsAg were quantified on days 4–7 post infection/transfection. (B) HepG2.2.15 cells, carrying an integrated HBV genome, were likewise treated with Am80. Secreted HBeAg and HBsAg were quantified at the end of the treatment period.

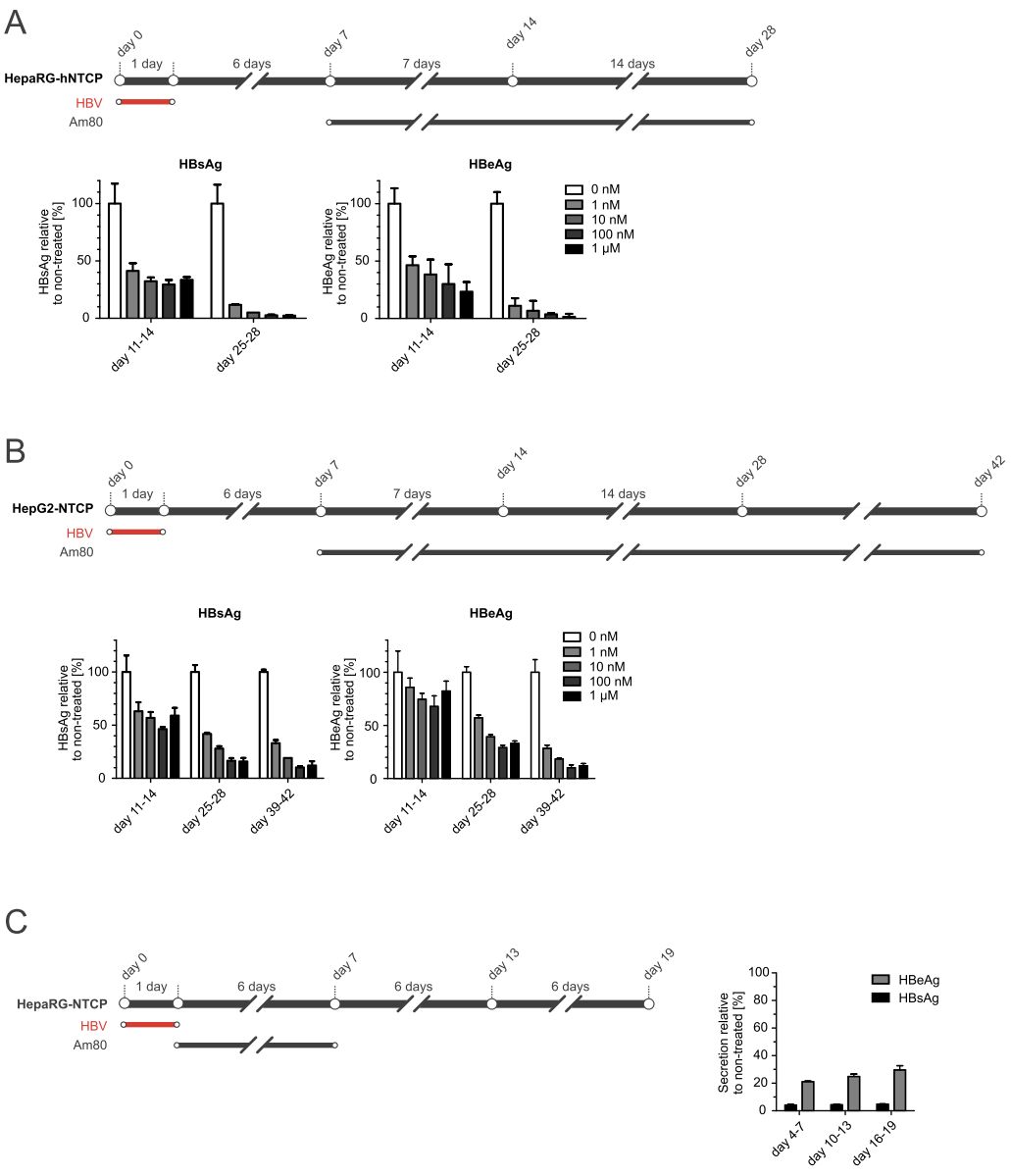

Fig. 7. Kinetics of RARα-mediated inhibition in authentic and hepatoma cell lines. (A) HepaRG-NTCP cells were infected with HBV and treated with the indicated concentrations of Am80 (0 nM up to 1 µM) between days 7 and 28 p.i. Secreted viral markers were assessed according to the scheme (days 11–14 and days 25–28). (B) HepG2-NTCP cells were similarly infected and treated. Viral markes were followed up until day 42 p.i. (C) Am80 (100 nM) was added directly post HBV inoculation for 6 days on HepaRG-NTCP cells. After withdrawal of Am80, secreted HBsAg and HBeAg were quantified after another 6, respectively 12 days, as depicted in the bar chart (right).

4. Discussion

We report the identification of RAR agonists with inhibitory activity against HBV infection, by using a two-arm screening of the Prestwick library. This infection-based screening approach assessed the effect on primary HBV (HBeAg, HBsAg and HBcAg) and HDV (HDAg) infection. It could also evaluate the released infectivity of HDV (HDAg secondary round infection). The positive control drugs, the entry inhibitor Myrcludex B (Gripon et al., 2005; Schulze et al., 2010) and the prenylation inhibitor Lonafarnib (Ledgerwood, 2015), as well as the nucleotide analogue Tenofovir as a negative control drug, showed the expected effects. At 10 µM, most drugs had no apparent effect on HBV infection. Several identified drugs have been reported previously in context of HBV/HDV infection: Cycloheximide reduces intracellular HBV DNA in HepG2.2.15 cells (Guo et al., 2009) and secreted HBV DNA (Lamontagne et al., 2013). Chicago sky blue 6B interferes with NTCP with an IC_{50} of 7.1 µM (Donkers et al., 2017). The appearance of these drugs on the list of top hits in our HBV screening indicates its integrity and reliability.

We newly identified Acitretin, displaying a moderate activity. Primary HDV infection was hardly inhibited, but released HDV infectivity was reduced, correlating to the reduction of HBsAg. This indicates that the described two-step infection can identify inhibitors targeting HBsAg secretion besides *de novo* HDV replication. Surprisingly, the effects of Acitretin and Am80 were much stronger in differentiated hepatic cells such as PHH and HepaRG-NTCP than in HepG2-NTCP cells. These differences may be due to either a lower activity of certain drugs affecting cellular host factors in hepatoma cells, or an enhanced sensitivity to certain drugs in artificially differentiated hepatocytes in *in vitro* cultures.

With a different screening approach employing PHH, a recent study also identified RAR agonists as inhibitors of HBV transcription from viral cccDNA (Li et al., 2018). In that study, the RARβ/γ-specific agonist Tazarotene reduced HBsAg with IC_{50}s < 30 nM in PHH. This is consistent with and partially confirms our results. However, that study did not implement the RARa-specific agonist Am80. We here report that the RARα-specific agonist Am80 is more potent than agonists addressing other RAR subtypes. Li et al. further report that co-treatment of Tazarotene with the RARα-specific agonist Ro41-5253 did not reduce Tazarotene activity; in our study, Ro41-5253 did not affect HBeAg and HBsAg levels (supplementary Fig. A4). When directly comparing Tazarotene and Am80 in PHH, we determined IC_{50}s of 39 nM for

Tazarotene, which is consistent with the described data, and 8.4 nM for Am80 with respect to HBsAg, suggesting that Am80 is around 5-fold more potent than Tazarotene, and that generally RARα may be more important for HBV transcription regulation than other retinoic receptors.

Am80 differs from other retinoids by two important means. First, it binds with high affinity and very selectively to RARα. Less selective compounds might even lessen their HBV-inhibitory effect exerted through RARα by additional activation of other retinoic receptors, such as RXR/PPAR (Huan et al., 1995). Second, Am80 has only a low affinity to the cellular retinoic acid binding protein (CRABP) (Chaudhuri et al., 1999). It is therefore less sequestered to CRABP, less retained in the cytosol than other retinoids, and may thereby be more available in the nucleus for interaction with RARα.

The RAR antagonist Ro41-5253 did not impair HBV infection in our study. By contrast, a previous study with HepaRG cells and PHH described a reduction of NTCP expression and thereby HBV infection at 10 μM by Ro41-5253 (Tsukuda et al., 2015). It is noteworthy that HepaRG-NTCP cells, used in this study, constitutively express high levels of NTCP under the EF-1α promoter (Ni et al., 2014). Unlike the endogenous NTCP promoter, EF-1α is apparently not negatively regulated by Ro41-5253, as shown by Tsukuda et al.

Consistent with our findings, a limited inhibition of HBV infection by the RXR agonist Bexarotene was recently described (Song et al., 2018). Additionally, the RXR antagonist PA452 inhibited secreted HBV markers at approximately 10 μM, consistent with recent observations (Xia et al., 2017). How exactly both RXR agonism and antagonism affect HBV infection is yet to be investigated.

The nuclear receptors RAR and RXR form heterodimers with each other, but also with other receptors, to regulate transcription. RXR in complex with the peroxisome proliferator-activated receptor (PPAR) has been shown to transactivate the HBV enhancer 1 element (Huan et al., 1995; Reese et al., 2013) in transfection experiments; this property of RXR/PPAR is independent from RAR. In contrast to transactivation by those nuclear receptors found in transfection experiments, we have found a pronounced inhibition of authentic HBV infection by RARα. In our study, transcription in the infection setting was reduced in a time-dependent and persistent manner, while in a transfection setting as well as from HepG2.2.15 cells, it was not affected. We hypothesize that Am80 inhibits transcription from cccDNA by a direct (regulation of transcription factors) or indirect (epigenetic silencing) manner.

In summary, we identified RARα as a potential new cellular target for HBV therapy. In particular, we identified Am80 (Tamibarotene), an already approved 3rd-generation retinoid, as the most potent inhibitor, selectively impairing cccDNA-mediated RNA transcription by a not yet well-understood mechanism. This provides the basis for further mechanistical studies, lead-compound development and may allow a fast translation into clinical applications in HBV-infected patients.

Conflicts of interest

S.U. holds patents and intellectual property rights for Myrcludex B, the entry inhibitor used in this study. All other authors declare no competing interests.

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Supplemental A. Further information about materials and methods used

A.1 HBV and HDV infection: Heparin-purified virus stocks prepared from supernatant of HepAD38 cells were used for HBV infection (multiplicity of genome equivalents 100) (Ni and Urban, 2017). For comparison of different HBV genotypes, a heparin-purified serum of a genotype B-infected patient was used; genotypes D and E were produced by transfection of HuH7 cells with plasmids containing 1.1-fold copies of the HBV genome, p26 (Ni et al., 2010) and pcDNA3.1 Zeo HBV1.1 gtE2 (the inserted HBV genome was kindly provided by Stefan Schäfer, University of Rostock). Supernatants of transfected cells were concentrated 50- to 100-fold by PEG precipitation prior to infection (Ni and Urban, 2017). HDV was produced by co-transfection of HuH7 cells with the plasmid pJC126, containing 1.1 copies of the HDV antigenome (Gudima et al., 2002), and the plasmid pT7HB2.7, coding for a 2.7 kb HBV genome encoding the three surface proteins (Sureau et al., 1994). Cell culture supernatants were enriched for HDV by heparin affinity chromatography (Lempp et al., 2016). Cells were inoculated overnight with HBV and/or HDV in medium containing 4% PEG and 1.5%–2.5% DMSO as described (Ni and Urban, 2017).

A.2 Quantification of viral RNA, HBV total DNA and cccDNA: For HBV RNA quantification, RNA from infected cells was extracted using the NucleoSpin RNA kit (Macherey Nagel). RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and the following primers: pregenomic RNA: forward CTCCTCCAGCTTATAGACC and reverse GTGAGTGGGCCTACAAA. Total RNA: forward TCAGCAATGTCAACGA CCGA and reverse TGCGCAGACCAATTTATGCC. GAPDH: forward GTGAACCATGAGAAGTATGACAAC and reverse CATGAGTCCTTCCAC GATACC. For HBV total DNA quantification, DNA from infected cells as well as from supernatants of infected cells was extracted using the NucleoSpin tissue kit (Macherey Nagel). Quantitative PCR was performed with the iTaq Universal SYBR Green Supermix (Bio-Rad) and the primers HBV total DNA: forward GTTGCCCGTTTGTCCTCTAATTC and reverse GGAGGGATACATAGAGGTTCCTTGA. For cccDNA quantification, a pre-treatment step with T5 exonuclease digestion was used to ensure the specific detection of cccDNA by PCR (Qu et al., 2018). In short, cells were harvested and DNA was extracted with the NucleoSpin tissue kit (Macherey Nagel). DNA samples were treated with T5 exonuclease, removing linear DNA and rcDNA, but not cccDNA. Quantitative PCR was performed using PerfeCTa qPCR ToughMix (Quanta Biosciences) with probe FAM-AGTTGGCGAGAAAGTGAAAGCCTGC-TAMRA, forward primer GTGGTTATCCTGCGTTGAT and reverse primer GAGCTGAGGCGGTATCT. For normalization, human beta globin from DNA samples before the T5 exonuclease treatment was quantified using iTaq Universal SYBR Green Supermix (Bio-Rad) with forward primer CAGGTACGGCTGTCATCACTTAGA and reverse primer CATGGTGTCTGTTTGAGGTTGCTA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.04.009.

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