Antiviral Research 169 (2019) 104538

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Anti-HBV activity of retinoid drugs in vitro versus in vivo

Gabriel Birkus[b], Chelsea Snyder[a], Robert Jordan[a], Tetsuya Kobayashi[a], Ryan Dick[a], Vlad Puscau[a], Li Li[a], Ricardo Ramirez[a], Madeleine Willkom[a], Yoshida Morikawa[c], William E. Delaney IV[a], Uli Schmitz[a,*]

[a] Gilead Sciences, 333 Lakeside Drive, Foster City, CA, 94494, USA
[b] IOCB, Flemingovo nám. 542/2, 160 00, Praha 6, Czech Republic
[c] Phoenix Bio, 3-4-1, Kagamiyama, Higashi-Hiroshima City, 739-0046, Japan

A B S T R A C T

We describe here the anti-HBV activity of natural and synthetic retinoids in primary human hepatocytes (PHHs). The most potent compounds inhibited HBsAg, HBeAg, viral RNA and DNA production by HBV infected cells with EC_{50} values ranging from 0.4 to 2.6 μM. The activity was independent of PHH donor and HBV genotype used in testing. 13-cis retinoic acid (Accutane) was selected for further evaluation in the PXB chimeric mouse model of HBV infection at doses allowing to achieve Accutane peak serum concentrations near its antiviral EC_{90} and exposures ~5-fold higher than a typical clinical dose. While these supraclinical exposures of 100 mg/kg/day were well-tolerated by regular Balb/c mice, PXB mice were more sensitive and even a lower those of 60 mg/kg/day led to significant weight loss. Despite dosing at this maximal tolerated dose for 28 days, Accutane failed to show any anti-HBV activity. RAR target engagement was verified using transcriptome analysis of liver samples from treated versus vehicle groups. However, gene expression changes in PXB liver samples were vastly muted when compared to the in vitro PHH system. When comparing transcriptional changes associated with the conditioning of fresh hepatocytes toward enabling HBV infection, we also observed a large number of changes. Noticeably, a significant number of genes that were up- or down-regulated by the conditioning process were down- or up-regulated by HBV infected PHH treatment with Accutane, respectively. While the lack of efficacy in the PXB model may have many explanations, the observed, opposing transcriptional changes upon conditioning PHH and treating these cultured, HBV-infected PHH with Accutane allow for the possibility that the PHH system may yield artificial anti-HBV hits.

1. Introduction

Hepatitis B Virus (HBV) is an enveloped DNA virus belonging to the Hepadnaviridae family with tropism for human hepatocytes (Beck and Nassal, 2007). More than 240 million individuals worldwide are chronically infected with HBV and about 800,000 die every year due to diseases complications such as liver cancer and cirrhosis. During infection, HBV's partially double-stranded relaxed circular DNA (rcDNA) genome is converted into covalently closed circular DNA (cccDNA), which persists as a nucleosome-bound mini-chromosome. cccDNA is the template for viral RNA transcription yielding pregenomic viral RNA (pgRNA) and mRNAs for HBV proteins, including the secreted HBV s- and e-antigen (Nassal, 2015). pgRNA also encodes capsid (core) protein and reverse transcriptase (P protein), and at the same time serves as a template for synthesis of rcDNA by P protein after it is incorporated into capsid particles (Bartenschlager and Schaller, 1992; Hirsch et al., 1990).

Treatments for infected individuals include nucleos(t)ide analogs and pegylated (PEG)-IFN. Nucleos(t)ide analogs act by inhibiting the reverse transcription of pgRNA, such that virions are not produced and consequently new cccDNA is no longer formed. Theoretically, however, a single copy of remaining cccDNA could reactivate a full infection (Nassal, 2015). Current nucleos(t)ide antivirals have no effect on HBV gene expression from cccDNA existing from the pre-treatment period, so relapse after nucleos(t)ide cessation is imminent (Kang et al., 2015). A finite 48 week therapy with PEG-INF can induce sustained viral

Abbreviations: ATRA, all-trans retinoic acid; Accutane, 13cis-retinoic acid; cccDNA, covalently closed circular DNA; HBeAg, HBV e antigen; HBsAg, HBV s antigen; HNF4α, Hepatocyte nuclear factor 4 alpha; pgRNA, pregenomic viral RNA; PHH, primary human hepatocytes; RAR, Retinoic acid receptor; rcDNA, relaxed circular DNA; RXR, Retinoid X receptor; SHP, small heterodimer partner

* Corresponding author.
E-mail address: uli.schmitz@gilead.com (U. Schmitz).

https://doi.org/10.1016/j.antiviral.2019.104538
Received 13 February 2019; Received in revised form 20 May 2019; Accepted 17 June 2019
Available online 19 June 2019

suppression in about 25% treated patients; however PEG-INF has poor tolerability (Kwon and Lok, 2011).

Only a small percentage of chronically-infected HBV patients achieve a functional cure, indicated by HBV S antigen (HBsAg) seroconversion following treatment with currently approved agents (Ezzikouri et al., 2014; Guidotti et al., 2015; Levrero et al., 2016; Li et al., 2015; Uchida et al., 2017; Ward et al., 2016). A sterilizing cure would require elimination of all cccDNA in infected hepatocytes, but may also necessitate elimination of cells harboring integrated HBV genomes. In contrast, functional cure is mediated by the host immune system and while not sterilizing, is able to exert long-lasting control over the virus. Additional mechanisms such as silencing of cccDNA and HBV gene expression may also be involved in functional cure. Immune exhaustion is likely to be a major barrier to functional cure and is likely driven by the constant presence of viral antigens, even during antiviral therapy (Wilson et al., 2016).

Unfortunately, there are still significant gaps in our knowledge of the persistence and regulation of HBV cccDNA transcriptional activity is still limited, despite a substantial amount of earlier work linking parts of host transcriptional machinery to HBV replication (Beck and Nassal, 2007; Zhang and Hu, 2015). Following the hypothesis that there may be druggable host factors involved in cccDNA dependent gene expression (Baumert et al., 2015; Li et al., 2015; Quasdorff and Protzer, 2010), we set out to screen a focused library of 250 pharmacological agents known to target proteins involved with host transcriptional regulation, including transcription factors, epigenetic regulators and DNA repair enzymes. To mimic the natural situation of a chronically infected human liver, we chose to evaluate our library in primary human hepatocytes (PHH) infected with HBV. The PHH assay system allows for stable HBV infection in a variety of donors using different HBV isolates and enables measurement of secreted antigens as well as intracellular viral RNA and cccDNA. Our screening efforts yielded several classes of transcriptional modulators with potent anti-HBV activity. The first class consists of retinoic acid receptor (RAR) agonists, exemplified by the FDA-approved drugs, all-trans retinoic acid (ATRA, Tretinoin) and 13cis-retinoic acid (Isotretinoin, Accutane). Another class comprises inhibitors of histone lysine demethylase 5 (KDM5) (WO2016/168349), which will be discussed in a separate publication.

We describe here characterization of anti HBV activity of retinoids in vitro and in vivo and our efforts to understand the discrepancy between the antiviral activity of Accutane on HBV-infected PHH and lack thereof in a transgenic mouse model of HBV infection. We describe how transcriptional profiling was used to come to an unexpected hypothesis by which retinoids counteract transcriptional changes in PHHs that are essential for their longevity in cell culture and their infectability with HBV..

2. Methods

2.1. Ethics statement

Primary human hepatocytes (PHH) isolated from deceased donor livers were purchased from Thermo Fisher Scientific (Waltham, MA), Lonza (Basel, Switzerland), BioreclamationIVT (Westbury, NY), and Corning, Inc. (Corning, NY). Consent was obtained from the donor or the donor's legal next of kin for use of the tissue and its derivatives for research purposes using IRB-approved authorizations. Plasma from CHB patients was purchased from Proteogenex (Culver City, CA) or BioCollections Worldwide, Inc (Miami, FL). Consent was obtained from the donor for use of the sample for research purposes using IRB-approved authorizations. All animal work was performed by Covance, Inc. (Princeton, NJ) or Phoenix Bio Inc. Studies in nonclinical species were conducted at test sites fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures in the protocol were in compliance with applicable animal welfare acts and were approved by the local Institutional Animal Care and Use Committee (IACUC). An attending laboratory veterinarian was responsible for providing the medical treatment necessary to prevent unacceptable pain and suffering for the animals on study.

The animal protocols and all procedures involving animals were approved by the Animal Welfare Committee of Covance or Animal Welfare Committee of Phoenix Bio and Covance adhered to the Guide for the Care and Use of Laboratory Animals. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

2.2. Compounds

Compounds **1, 2, 3, 5, 7, 8, 10, 15, 16** and **17** were purchased from MilliporeSigma (St. Louis, MO, USA). Compounds **11, 12, 13** and **14** were purchased from Tocris Bioscience (Minneapolis, MN, USA). Compounds **18, 19,** and **20** and **14** were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Compounds **4, 6** and **9** were obtained from Calbiochem (St. Louis, MO), eNovation Chemicals LLC (Bridgewater, NJ, USA) and Xcessbio Biosciences Inc. (San Diego, CA, USA), respectively.

2.3. PHH screening protocol

HBV antiviral activity was assessed in a 96-well viral replication assay using primary human hepatocytes (PHH) from a single donor 8181 (PHH) which were infected with a P21 virus (genotype A). Production of secreted HBV s- and e-antigens evaluated after 9 days p.i. . PHH (Life Technologies, Chicago, Il) were plated at density 65,000 cells per well on collagen coated tissue culture plates using Plating Media containing William's Medium E supplemented with 1% Penicillin/Streptomycin, 4 μg/mL human recombinant insulin, 2 mM glutamax, 15 mM Hepes, 1 μM Dexamethasone and 5% fetal bovine serum (Life Technologies, Cat#A12176-01 Life Technologies, Chicago, Il). After a 4-h incubation at 37 °C, cells were switched to Maintenance Media (Cat#CM4000-A15564 Life Technologies, Chicago, Il) containing William's Medium E supplemented with 0.5% Penicillin/Streptomycin, 6.25 μg/mL human recombinant insulin, 6.25 μg/mL human transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, 5.35 μg/mL linoleic acid, 2 mM glutamax, 15 mM Hepes, 0.1 μM dexamethasone, 2% fetal bovine serum, and 2% DMSO (Cat#D2650 Sigma, St. Louis, MO). On the next day, cells were infected with approximately 500 genome equivalent of selected HBV clinical isolates (21P (GTA), 32P (GTA), 91P (GTA), AD38 (GTD), 65P (GTD) or 30P (GTE); ProteoGenex, Culver City, CA) per cell in Maintenance Media supplemented with 4% PEG 8000 (Cat#V3011 Promega, Madison, WI). After 24 h incubation, cells were washed three times with William's Medium E and fed with fresh Maintenance Media. Small molecule inhibitors targeting RARs were serially diluted in Maintenance Media and added to cells at 3 days post infection (p.i.). Media with compounds was replenished every 3–4 days. Media collected on various days was used for determination of HBsAg and HBeAg levels by MSD ELISA, and HBV DNA by qPCR. All data were converted into percentages of the untreated control and non-linear regression was performed to calculate EC_{50} or CC_{50} values.

2.4. Determination of HBV viral DNA

Collected medium (20 μL) was treated with 1 U of Turbo DNAse (Life Technologies, Chicago Il #AM2239) for 60 min at 37 °C following the protocol recommended by the manufacturer. After DNAse was inactivated by addition of EDTA to the final concertation of 15 mM, viral DNA from virions was further purified using DNeasy 96 Blood & Tissue Kit (Qiagen, Cat# 69582) following the manufacture protocol. HBV DNA levels were measured by qPCR using the FastAdvanced Taqman Mix (Life Technologies, Chicago Il, Cat# 4444557) with primers specific to the HBx region (forward: 5′-CCG TCT GTG CCT TCT CAT CTG-

3′, reverse: 5′-AGT CCA AGA GTY CTC TTA TGY AAG ACC TT-3′, probe: 5′-FAM-CC GTG TGC ACT TCG CTT CAC CTC TGC-BHQ1-3′). HBV pHY plasmid, containing 1.0 copy of EcoR I digested genotype A (ADW2) HBV genome cloned in pBluescript(+) was used as a standard. HBV DNA copy numbers were calculated from Ct values and standard curve and then were expressed as a percentage of the untreated control to calculate EC_{50}s.

2.5. Cell viability assay

Alamar Blue cell viability reagent (Cat#DAL1100 Life Technologies, Chicago, Il) was diluted 1 to 10 in pre-warmed Maintenance Media and added to the cells. Cells were incubated for 4 h at 37 °C and the fluorescence signal, which is proportional to the number of metabolically-active cells, was read using a fluorimeter with excitation/emission spectra set at 560/590 nm, respectively. Data were converted into percentages of the untreated control and non-linear regression was performed to calculate CC_{50} values.

2.6. Determination of HBV viral RNA

Following the Alamar Blue measurement, media was removed and total RNA from the cells was isolated using the RNeasy 96 Kit (Qiagen, Venlo, Netherlands, Cat#74182). HBV mRNA levels from total RNA isolations were measured by RT-qPCR using the TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Chicago Il, Cat#4444436) with primers specific to the HBx region (forward: 5′-CCG TCT GTG CCT TCT CAT CTG-3′, reverse: 5′-AGT CCA AGA GTY CTC TTA TGY AAG ACC TT-3′, probe: 5′-FAM-CC GTG TGC ACT TCG CTT CAC CTC TGC-BHQ1-3′) that should amplify all four HBV mRNA transcripts. GAPDH mRNA levels were also measured by RT-qPCR to control for differences in cell number, toxicity, and RNA purification efficiency (Cat#4390849 Life Technologies, Chicago Il). HBV mRNA Ct values were normalized using their cognate GAPDH mRNA Ct values by the delta-delta-Ct calculation and then expressed as a percentage of the untreated control.

2.7. HepG2-NTCP assay

NTCP-HepG2 cells infected with AD38 virus were seeded on 384-well collagen coated plates at a density of 20,000 cells/well containing serially diluted small molecule inhibitors or DMSO (0.5%) in a final volume of 80 µl. After an incubation time of 5 days, the supernatant was incubated with lysis buffer Waltham, MA, Proteinase K (Affymetrix, Santa Clara, CA) and an HBV specific probe set (Affymetrix, Santa Clara, CA), followed by addition of 0.2M NaOH to denature the DNA and addition of neutralization buffer (Affymetrix, Santa Clara, CA). The resulting lysed and neutralized supernatant was then transferred to 384-well plates coated with capture oligos and incubated overnight. Following the overnight incubation the wells were incubated for 1 h sequentially with a pre-amplifier, amplifier and labeled probes conjugated to alkaline phosphatase with wash steps between incubations. After the final wash step the alkaline phosphatase substrate (Luminol APS5) was added and the resulting luminescence signal was read on an Envision Plate Reader (PerkinElmer). EC_{50} values were calculated from the fit of the dose − response curves to a four-parameter equation.

The HBsAg and HBeAg concentration in the culture supernatant was measured using an HTRF assay (Cisbio, Bedford, MA). Briefly, a mixture of two conjugated anti-HBsAg antibodies (anti-HBsAg antibodies XTL17 conjugated with Terbium and XTL19 conjugated with d2 receptor [need good reference]) was mixed with 3 µL of culture supernatant and the plates were read in an Envision plate reader at emissions of 665 nm and 615 nm. The ratio of 665/615 was used to plot compound dose response curves.

A similar method was used to measure HBeAg concentration. The anti-HBeAg antibodies used were GWB-F19420 (conjugated with terbium) and GWB-24DEF (conjugated with d2 receptor) [need reference]. EC_{50} values were calculated from the fit of the dose − response curves to a four-parameter equation.

2.8. MT4 cytotoxicity assay

Compound cytotoxity was evaluated in a 5 day assay using MT4 cell as described previously (Fenaux et al., 2016).

2.9. Metabolic stability of compounds in PHH

A fixed amount of test compound (10 µM) was incubated with PHH from donor 8181 for 3 days with and without aminobenzotriazole (ABT) at 1 mM. The fraction remaining was determined via mass spectroscopy comparing amounts at the end of day 3 versus t = 0.

2.10. DMPK studies

Studies were performed in male Balb/c or PXB mice. All animals received an oral dose of the dose formulation via gavage twice a day. Blood sampling was from the retroorbital sinus under isoflurane anesthesia Blood samples were anticoagulated with K_2EDTA and plasma prepared by centrifugation. Plasma proteins were precipitated with acetonitrile containing the assay internal standard (labetalol). Aliquots of the supernatant were subject to chromatography on a BDS Hypersil C8 column (50 × 2.1 mm, 5 µm particle size) with an Agilent 1200 Series HPLC. Mobile phase contained 0.1% (v/v) formic acid and 1% (v/v) 2-propanol and elution was achieved by a gradient of acetonitrile in water. Detection was with an API-6500 triple quadrupole mass spectrometer with an electrospray interface operating in positive ionization mode. Quantification was by analyte/internal standard peak area ratios determined through multiple reaction monitoring and by reference to extracted standards and quality control samples. The detection limits were 10 nM for isotretinoin and 4 nM for the 4-oxo metabolite.

2.11. In vivo pharmacokinetic and tolerability study in BALB/c mice

Male BALB/c (BALB/cAnNHsd) mice were obtained from Envigo, RMS, Inc. Animals were acclimated, but not fasted, prior to dose administration. Dose formulations were administered twice daily for 7 days by oral gavage at a dose volume of 5 mL/kg. Animals were divided into groups receiving dose levels of either 0 (vehicle consisting of 9% propylene glycol, 13.3% ethanol, 33.3% Labrasol, and 44.4% Kolliphor HS15), 25, 75 or 100 mg/kg/day Accutane. Body weights and clinical observations were recorded daily and blood samples were collected for bioanalysis and clinical chemistry. Clinical chemistry samples were collected via cardiac puncture into serum separator tubes, 24 h after the last dose administered. Serum glucose, triglycerides, cholesterol, total protein, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma glutamyltransferase and calcium were measured on a Roche Modular Analytics (Roche Diagnostics Corporation, Indianapolis, IN) analyzer using the manufacturer's applications. Animals were euthanized at the end of the study via exsanguination under isoflurane anesthesia, and liver samples were collected and flash frozen for gene expression analysis.

2.12. 2.12 In vivo pharmacokinetic and tolerability study in PXB-mice

This in vivo experiment was performed in strict accordance with the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Ethics Committee at PhoenixBio Co., Ltd., and appropriate efforts were made to reduce animals suffering. Male PXB-mice from PhoenixBio Co., Ltd. were 14–21 weeks old at the start of the study. Dose formulations were administered twice daily for 14 days via oral gavage at a dose level of 2.175 mL/kg. Animals were divided into two groups receiving

dose levels of either 0 (vehicle consisting of 10.3% propylene glycol, 38.5% Labrasol, and 51.3% Kolliphor HS15) or 60 mg/kg/day Accutane. Body weights and clinical observations were recorded daily and blood samples were collected for bioanalysis and measurement of human albumin.

2.13. PXB mouse efficacy model

Human liver-chimeric uPA/SCID mice, PXB mice were generated by PhoenixBio Co., Ltd. as described (Tateno et al., 2015; Tsuge et al., 2005). Human hepatocytes were from donor BD195 (BD Biosciences, Woburn, MA). HBV infection was performed with an HBV genotype C virus preparation (PhoenixBio), and allowed to proceed for at least 8 weeks to establish persistent infection of the human hepatocytes PXB chimeric liver. Mice were 20–22 weeks of age at the time of randomization. HBV-infected mice were randomized into vehicle and treatment groups based on body weight, blood human albumin (h-Alb), and serum HBV DNA concentrations. All mice had blood h-Alb levels between 9.3 and 12.6 mg/mL and serum HBV DNA levels above 10^8 copies/mL with a range from 1.6×10^8 to 3.9×10^8 copies/mL. Mice received Accutane 30 mg/kg twice daily or dosing vehicle (vehicle control) twice daily, vehicle was the same as in 2.16. Blood was drawn under isofluorane anesthesia at the indicated times via the retroorbital plexus/sinus. Terminal blood collection was done under anesthesia via the heart. Livers were collected, weighed, and separated into pieces that were flash frozen in liquid nitrogen, or immersed in RNAlater solution (Life Technologies, Carlsbad, CA) or formalin-fixed and paraffin-embedded. The handling and all experimental procedures using live animals for these studies had been approved by the Animal Ethics Committee of PhoenixBio.

2.14. RNAseq analysis

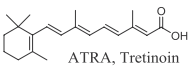
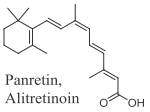
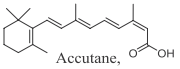
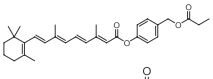
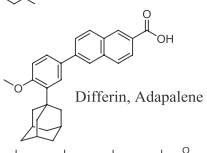
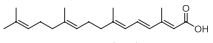
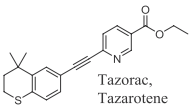
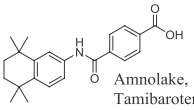
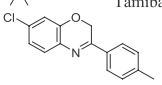
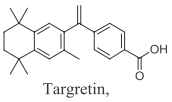
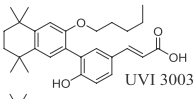
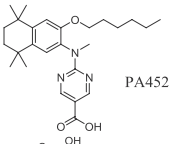
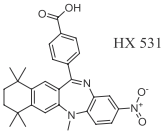
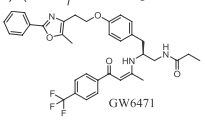
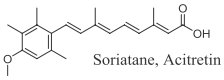
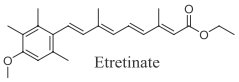
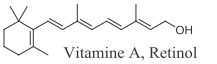
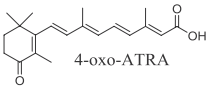
Cultured PHH cells and PXB liver samples were preserved in RNALater before extraction with [Qiagen Kit Cat No.: 76104]. Total RNA was assessed with an Agilent Bioanalyzer and all samples obtained RNA Integrity Numbers above 9.0. Poly(A) enriched mRNA libraries were created using TruSeq Stranded mRNA Library Prep kit for Illumina HiSeq sequencing with a minimum depth of 50 million reads per sample. Due to the humanized livers in PXB mice, we developed a pipeline to analyze RNA-Seq reads in human and mouse genomes. Reads were aligned to a combined reference of human hg38 and mouse mm10 genomes using STAR and assigned to gene annotations using featureCounts. Normalization of gene counts and differential expression estimations were completed in R using the limma package. Key Pathway Advisor (Clarivate Analytics) (Dubovenko et al., 2017) was carried out using genes with at least ± 2 fold change after retinoid treatment. We used Gene Set Enrichment Analysis to assess changes in gene set collections C2 and C3 from MSigDB (Subramanian et al., 2005).

3. Results

3.1. Antiviral activity

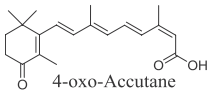
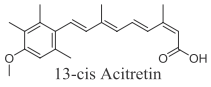
Natural and synthetic retinoids used in our study are listed in Table 1 along with their primary mode of action and their clinical status. Our panel includes both, agonists and antagonists for RAR and RXR (Benbrook et al., 2014; di Masi et al., 2015). In addition, retinoids **15** and **16**, are tight binding ligands for the cellular retinoic acid binding protein (CRABP) (Norris et al., 1994), thus thought to be increasing the intracellular stores of free all-trans retinoic acid (ATRA, **1**). Compounds were tested in a viral replication assay where production of secreted HBV s- and e-antigens was evaluated after 9 days p.i. (HBsAg-EC_{50}, HBeAg EC_{50} in Table 2). For active compounds without toxicity we also measured the reduction of secreted HBV DNA and intracellular

Table 1
The structure and name of the compounds, mode of action and their clinical application.

Entry	Structure and Name	Clinical Application
1[a]	 ATRA, Tretinoin	FDA 1962, APL/oral
2[a]	 Panretin, Alitretinoin	FDA 1999 Kaposi's sarcoma /oral
3[a]	 Accutane, Isotretinoin	FDA 1982, Acne/oral
4[a]		NA
5[a]	 Differin, Adapalene	FDA 1996, acne /topical skin
6[a]	 Peretinoin	PIII (2015), HCC/oral
7[a,c]	 Tazorac, Tazarotene	FDA 1996, Psoriasis /topical skin
8[a,d]	 Amnolake, Tamibarotene	FDA. 2005, APL/oral
9[e]	 AR7	preclinical
10[f]	 Targretin, Bexarotene	FDA. 1999, cancer/oral
11[g]	 UVI 3003	preclinical
12[g]	 PA452	preclinical
13[g]	 HX 531	preclinical
14[g]	 GW6471	preclinical
15[h]	 Soriatane, Acitretin	FDA. 1989, psoriasis/oral
16[h]	 Etretinate	FDA 1986, Removed psoriasis/oral
17[i]	 Vitamine A, Retinol	Vitamin A
18[j]	 4-oxo-ATRA	preclinical

(*continued on next page*)

Table 1 (*continued*)

Entry	Structure and Name	Clinical Application
19[k]	 4-oxo-Accutane	preclinical
20[l]	 13-cis Acitretin	preclinical

[a] RAR and RXR agonist,.
[c] RAR beta and gamma selective agonist.
[d] RAR alpha selective agonist.
[e] RAR antagonist.
[f] RXR agonist.
[g] RXR antagonist.
[h] CRABP binder.
[i] RA precursor.
[j] ATRA metabolite.
[k] Accutane metabolite.
[l] Acitretin metabolite.

RNA (PHH HBV vDNA EC_{50}, HBV vRNA EC_{50}). To rule out that viral antigen reduction was not caused by toxicity, we used an Alamar Blue live cell staining assay (PHH CC_{50} in Table 2). The majority of compounds had antiviral activity that was not due to cellular toxicity. Since cultured PHH's retain their profound capacity to metabolize small molecules we expected that many compounds would be metabolized during prolonged exposure in the assay. Hence, we determined the fraction remaining for each compound (PHH Stability %-left in Table 2) after 3 days incubation with cells. In addition, we repeated our PHH assay in the presence of 1 mM aminobenzotriazole, ABT, a pan-Cytochrome P450 inhibitor (Emoto et al., 2010). Despite this, the lack of activity of some of compounds could not be interpreted because of their metabolic instability even in the presence of the inhibitor (data not shown).

As the inhibitory effect of retinoids on cell growth of dividing cells is well known (Kizaki et al., 1996), we also determined cellular toxicity in the MT4 cell line (MT4 CC_{50} in Table 2). In all cases, retinoids appeared more toxic toward MT4 cells, sometimes by several orders of magnitude, e.g. compound **1** and **10**.

In general, EC_{50} values for viral RNA and DNA reduction were 2-4-fold lower than those for HBsAg and HBeAg. Overall the RAR agonists, ATRA, **1**, 9-cis ATRA, **2,** Accutane, **3,** and synthetic RARα agonist tamibarotene, **8**, (Lefebvre et al., 1995), emerge as the compounds with the most profound overall anti-HBV activity, along with Acitretin, **15**, the potent CRABP binder. Other RAR agonists with promising anti-HBV activity were compounds **4**, a prodrug of ATRA, and the retinoid metabolites 4-oxo ATRA, **18**, 4-oxo-Accutane, **19**. Neither compound appears toxic in the PHH assay. In addition, RXR antagonists (compounds **11–14**) along with precursor of ATRA, retinol, **17,** had no activity in the PHH anti-HBV assay. RXR agonist bexarotene (compound **10**) was reported recently (Song et al., 2018) to inhibit HBV in HepG2-NTCP cells and Tupaia hepatocytes when added within the first 24hrs of infection. However, we found that bexarotene's anti-HBV activity in our PHH system was not distinguishable from its toxicity.

To be sure that the observed activity of retinoids was not PHH donor or virus dependent, we also evaluated activity of ATRA, **1** and Accutane, **3** in two extra donors PHH donors (donors 4239 and 8130) using six different clinical isolates of HBV virus (genotypes A, D and E). Table 3 shows that inhibition of HBsAg and HBeAg by ATRA and Accutane is indeed PHH donor and HBV virus independent.

At this point we wanted to assess if these observed antiviral activities could have a clinical utility for treating HBV. To that end we compared typical clinical exposures reported for the most promising retinoid drugs with our EC_{50}s. Note that these clinical retinoids are highly protein bound in plasma (> 99.9%) (Thatcher and Isoherranen, 2009; Urien et al., 1992) such that a protein-binding adjustment should be applied to the EC_{50}s. However, we found that these drugs were also highly protein bound in our cell culture medium (> 99%) and that addition of 50% human serum to our PHH medium did not significantly increase the EC_{50}s (data not shown). Therefore, we evaluated EC_{50}s without protein binding adjustment against typical retinoid drug exposures achieved in patients. For all drugs the reported clinical C_{max} is within twofold of our HBV EC_{50}s. Human plasma C_{max} for the natural retinoids ATRA and Accutane is 1.3 μM, and 1–3 μM, respectively (Gota et al., 2016; Thatcher and Isoherranen, 2009). The synthetic retinoid drugs are associated with somewhat lower exposures. Acitretin, used to

Table 2
Retinoids cellular activity.

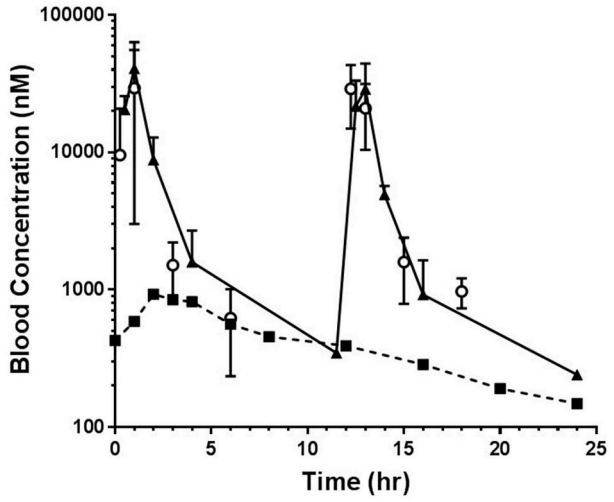
compound	HBeAg[a] EC_{50} (μM)	HBsAg[a] EC_{50} (μM)	HBV RNA[a] EC_{50} (μM)	HBV DNA[a] EC_{50} (μM)	PHH[a] CC_{50} (μM)	MT4[b] CC_{50} (μM)	PHH Stability (% left)	PHH +ABT Stability(% left)
1	1.2	1.7	0.4	0.4	> 100	0.17	26.2	24.2
2	0.6	1.1	1.2	0.6	> 100	0.37	34.5	28
3	0.6	2.6	1.2	1.2	> 100	20.8	30	nd
4	7.1*	11.9*	0.8	0.4	> 100	15.8	12.8	14.8
5	5.7	14.7	nd	nd	51	30.6	60.1	84.6
6	25.5*	33.2*	nd	nd	> 100	19.6	0	0
7	2.4*	22.8*	nd	nd	> 100	> 23	0.437	7.0
8	0.3	6.4	1.8	0.2	> 100	30.5	12.1	14.7
9	> 100	> 100	nd	nd	> 100	9.3	24.9	22.4
10	10.3	11.5	nd	nd	5.6	0.007	nd	nd
11	23.9	30	nd	nd	35.7	19.6	0.9	1.0
12	19.4	8.2	nd	nd	29.2	> 24	0.7	2.2
13	11.9	8	nd	nd	35	8.5	4.9	45.8
14	9	10.4	nd	nd	13	10.9	nd	nd
15	0.4	0.3	0.4	0.3	88	15.8	13	nd
16	4.8*	4.3*	nd	nd	> 100	20.1	nd	nd
17	> 40.0*	> 40.0*	nd	nd	13.5	17	20.9	31.2
18	8.6	8	nd	nd	> 100	2	nd	nd
19	5.48	8.14	nd	nd	> 100	8.4	nd	nd
20	2.61	4.4	nd	nd	> 100	47	nd	nd

[a] PHH donor 8181 was infected with patient virus p21 (MOI 500) for three days before serially diluted compounds 1–20 were added to the cells. Activity of the compounds was monitored on day 12 p.i. using HBsAg, HBeAg, vRNA, vDNA and cytotoxicity readout as described in Methods. The compounds and medium were replenished every 3–4 days in all experiments. Nd = not determined, * day 16 data.
[b] Cytotoxicity was determined on day 5.

Table 3
Effect of ATRA and Accutane on HBsAg and HBeAg across different virus genotypes and PHH donors.

Patient HBV viruses	ATRA				Accutane			
	HBsAg EC_{50} (μM)		HBeAg EC_{50} (μM)		HBsAg EC_{50} (μM)		HBeAg EC_{50} (μM)	
	PHH 4239	PHH 8130	PHH 4239	PHH 8130	PHH 4239	PHH 8130	PHH 4239	PHH 8130
21P (GTA)	0.8 ± 0.4	0.9 ± 0.5	1.1 ± 0.4	0.4 ± 0.2	3.2 ± 1.2	2.5 ± 1.0	3 ± 0.9	0.8 ± 0.3
32P (GTA)	0.6 ± 0.2	0.5 ± 0.2	0.7 ± 0.3	0.4 ± 0.1	2.3 ± 1.0	2.1 ± 0.8	2.6 ± 1.4	1.8 ± 0.4
91P (GTA)	0.6 ± 0.3	0.6 ± 0.2	0.7 ± 0.2	0.5 ± 0.3	2.3 ± 0.8	2.0 ± 1.0	2.1 ± 0.9	1.8 ± 0.5
AD38 (GTD)	0.4 ± 0.1	0.6 ± 0.4	0.5 ± 0.3	0.4 ± 0.2	1.9 ± 0.5	2.1 ± 0.4	1.4 ± 0.8	1.4 ± 0.8
65P (GTD)	0.6 ± 0.2	0.5 ± 0.3	0.6 ± 0.1	0.5 ± 0.2	2.0 ± 1.0	1.7 ± 0.6	2.1 ± 1.0	0.9 ± 0.3
30P (GTE)	0.8 ± 0.5	1.3 ± 0.5	0.6 ± 0.2	0.5 ± 0.2	2.3 ± 1.0	2.1 ± 1.2	1.9 ± 0.5	1.7 ± 0.6

PHH donors 4239 and 8130 were infected with patient viruses (MOI 500) for three days before serially diluted ATRA and Accutane were added to the cells. Activity of the compounds was monitored on day 18 p.i. using HBsAg and HBeAg readout. The compounds and medium were replenished every 3–4 days in all experiments. CC_{50}s for both compounds were determined by AlamarBlue method and were > 100 μM.



Dose Level	Cmax (uM)	AUC_{last}(uM·hr)
80 mg in Human	0.93	10
50 mg/kg BID in Balb/c Mice	40.7	114
30 mg/kg BID in PXB Mice	34	188

Fig. 1. Accutane exposure in Balb/c and PXB mice compared with a typical clinical exposure in Humans: Balb/c mice 50 mg/kg BID for 7 days (-▲-, day 7, each point reflects average of 3 mice), PXB mice 30 mg/kg BID for 28 days (-○-, day 28, each point reflects average of 4 mice) and a single 80 mg Human dose on day 25 after 24 days of 40 mg/kg BID dosing (-■-)(concentration time points were extracted from Colburn et al., 1983 Fig. 1 and reflect the mean of 10 patients).

treat severe resistant psoriasis, achieves a mean C_{max} of 1.2 μM (Pilkington and Brogden, 1992). Tamibarotene, approved in Japan since 2005 for treating APL, but still under development in the U.S. for Myelodysplasia, reaches a C_{max} of 0.3 μM (Kanai et al., 2014). This suggested that these retinoid drugs could have a potential clinical anti-HBV effect. However, we could not find any public record of HBV antigen reduction in HBV-infected patients using retinoids. When re-examining our older HBV clinical trial data [ClinicalTrials.gov Identifier: NCT00117676] we found three subjects that reported undergoing Accutane therapy while being treated with HBV nucleotide drugs. No reduction in HBsAg or HBeAG were recorded. Since this observation is merely anecdotal, we wanted to evaluate one of the retinoid drugs in the PXB mouse model of human HBV infection where potentially much higher than clinical doses could be used.

3.2. Accutane tolerability in mice

We needed to identify the drug that would give us the best chance to reach drug levels in PXB mice several fold higher than the relevant HBV EC50s. Using substantial clinical information available for the four drugs, including clinical DMPK properties and dose-limiting toxicity profiles, we chose Accutane for our efficacy study, as detailed in the following. Accutane is widely used for treating juvenile Acne and a typical dose is 40 mg, administered twice daily for several months, yielding exposures with C_{mean} of 1–3 μM (Thatcher and Isoherranen, 2009). Besides its profound mechanism based teratogenicity, dose limiting toxicities consist of hypercalcemia, combinations of skin and gastrointestinal discomfort, as well as hematopoietic toxicities. A maximum tolerated dose in pediatric patients has been reported as 2x80 mg/m^2/day, the equivalent of a daily 256 mg dose for an adult (Villablanca et al., 1995). Dose limiting toxicities are generally not targeted to the liver, although liver enzyme elevations have been reported, but were fully reversed after a few days (Gold et al., 1983). The other drugs are associated with more side effects, including hepatotoxicity, which would be a major obstacle for a potential clinical evaluation in HBV patients. Taken together, we aimed for Accutane peak serum concentrations near the antiviral EC_{90} (10–20μM), such that a proof of concept study in HBV patients could be pursued after demonstrating efficacy in the PXB mouse model of HBV infection.

Despite Accutane's broad clinical use, there are no published tolerability studies in rodents to define the maximum tolerated dose relevant for our PXB HBV study. Therefore, we conducted a 7 day tolerability study in Balb/c mice after determining the maximum achievable exposure via oral administration. The highest dose level in the study was 100 mg/kg/day Accutane, dosed 50 mg/kg BID for seven days by oral gavage in an organic solution., This dose level achieved a C_{max} of 40 μM and $AUC_{0\text{-}24h}$ of 558 μM*hr, which is ~40 fold and ~14 fold higher, respectively, when compared with the human exposure from a 80 mg qd dose (Colburn et al., 1983) (Fig. 1). To test tolerability, we evaluated three Accutane dose levels for 7 days (25, 75 and 100 mg/kg/day po via BID dosing). Only minimal weight changes were observed in the highest dose group when compared to vehicle controls (a 5% decrease in body weight gain, Fig. S1). There were no remarkable clinical signs or significant differences in a clinical chemistry panel when compared to vehicle controls, establishing that 100 mg/kg/day was a well-tolerated dose level in Balb/c mice. However, due to the fragility of the PXB mouse model, the highest tolerated dose level was only 60 mg/kg/day (given 30 mg/kg BID), when the tolerability study was repeated in PXB mice. Dose levels above 60 mg/kg/day resulted in mortality in PXB mice. At the end of this 14 day tolerability study, C_{max} and AUC for Accutane was 37 fold and 19 fold higher, respectively, when compared to exposure from an 80 mg dose in patients (Fig. 1). Based on the Accutane AUC, the dose level of 2x30 mg/kg/day achieves ~5 fold higher

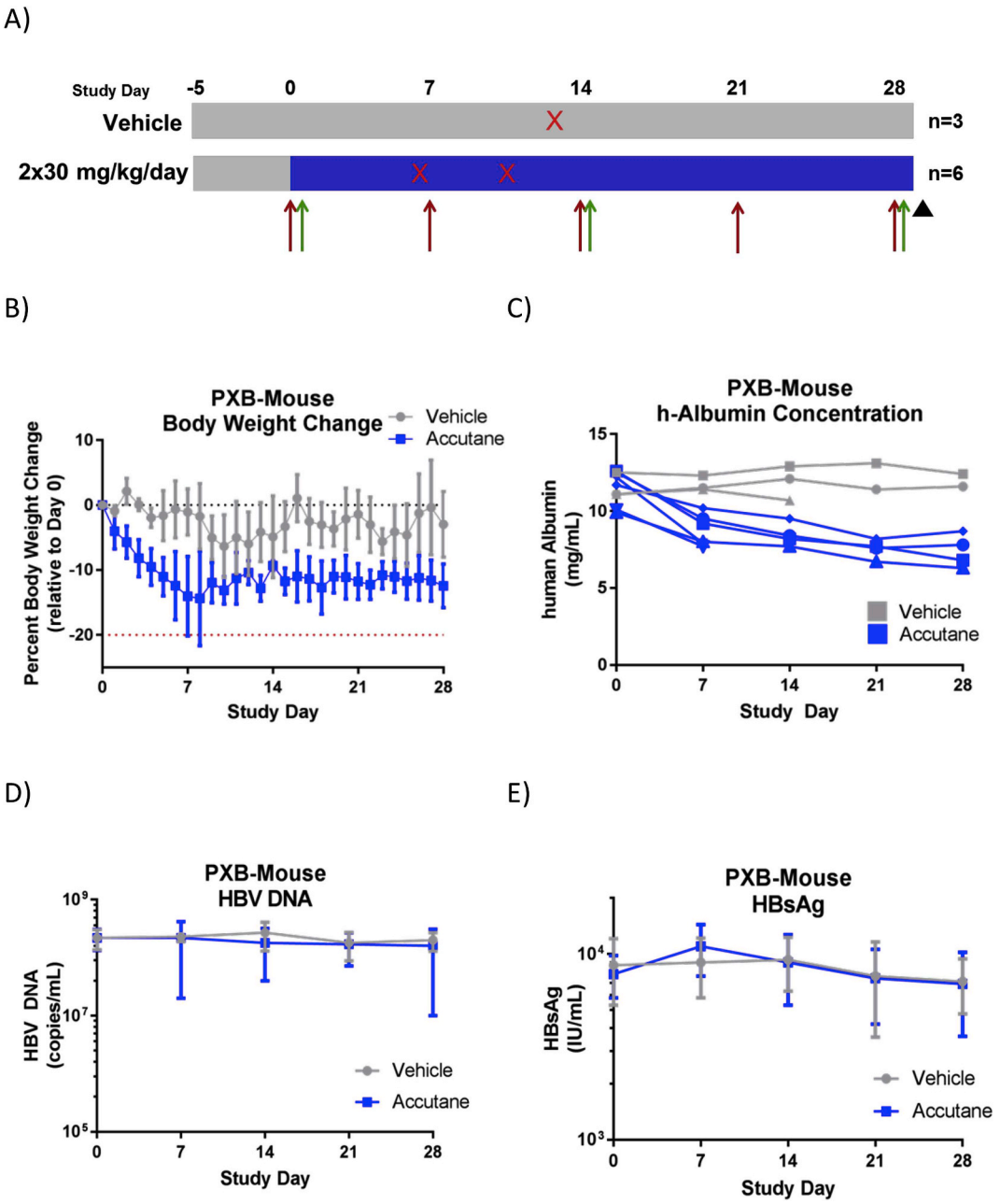


Fig. 2. Design and Results of the PXB mouse efficacy study: (A) study design (red arrows denote blood sampling days for albumin and viral titers, green arrows denote sampling days for DMPK; X denotes death); (B) average weight changes; (C) changes in human albumin production; (D) HBV DNA changes; (E) HBsAg changes.

exposure compared to the clinical human dose.

3.3. PXB mouse efficacy study

Before proceeding with our efficacy study, we confirmed the antiviral activity of Accutane in PXB derived hepatocytes (data not shown). Six PXB mice that had been stably infected with HBV genotype C, were treated twice a day with 30 mg/kg/day po for 28 days, while three mice in the control group were dosed twice daily with vehicle only. Since our vehicle produces a mild weight loss in PXB mice by itself, we dosed all mice with vehicle only for five days prior to starting treatment (Fig. 2a). Upon dosing Accutane, PXB mice showed progressive weight loss up to day 7, after which weights stabilized. Average weight loss in the treatment group was 15%, compared 6% in the vehicle control group. Two deaths occurred in the treatment group (day 7 and day 10) compared to one in the control group (day 13). Additionally, we noticed a slight Accutane-induced reduction of human Albumin in the PXB mice (Fig. 2b), which could also be a sign of general toxicity. Exposures of Accutane and its metabolite on day 28 were consistent with the 14 day study discussed above. These findings support that our 28 day PXB efficacy study evaluated Accutane near the highest tolerated dose. Despite the achieved drug exposure, no reduction was observed for serum HBV DNA and HBsAg, indicating that Accutane lacks anti-HBV activity in this in vivo mouse model (Fig. 2c).

3.4. RNAseq

To understand the discrepancy between in vitro and in vivo anti-HBV activity, we evaluated the impact of retinoids on global gene expression in both PHH and PXB liver tissue using RNAseq (Wang et al., 2009). For the in vitro PHH transcriptome analysis, cells were treated by either Accutane or ATRA for 8, 16 and 32 h at three dose levels,

ranging from the approximate EC_{50} to EC_{90}, with time-matched DMSO as control. Not surprisingly, gene expression changes were highly correlated between Accutane and ATRA (example dataset shown in Fig. S1). Overall drug concentration had a stronger effect on global gene expression profiles than the time of exposure, leaving the 32 h high concentration data set as the one with the most pronounced transcriptional changes. Using this representative data set for Accutane, we found that 428 genes are upregulated over 4-fold, while 197 genes are downregulated more than 4-fold. As expected, genes associated with metabolism feature prominently, CYP26A1 and CYP26B1 are among the most highly upregulated genes while CYP7A1 is the most downregulated (all over 1000-fold, see Table S1). Not surprisingly, our Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) identified a number of gene sets related to other studies using retinoids (supp. Table S1). A weaker GSEA hit spiked our interest as it tied together the effect of ATRA and the HBV-related liver specific transcription factor HNF4α by upregulation of SHP (gene set OHGUCHI_LIVER_HNF4A_TARGETS_DN in Table S2). Mamoon et al. showed that ATRA leads to early upregulation of SHP (gene name NR0B2) in AML12 cells (Mamoon et al., 2008, 2014). HNF4α was shown to enable HBV replication in HEK283T cells (Li et al., 2009; Oropeza et al., 2008) while additional expression of negative regulatory factor SHP reduces replication to undetectable levels. Furthermore, targeting HNF4α with shRNA leads to reduction of HBV replication in an earlier HBV mouse model (He et al., 2012). Thus, one putative hypothesis for the mechanism of the antiviral activity of retinoids in PHH is that they upregulate SHP expression, we observe 13-fold, which then sequesters HNF4α (unchanged) at the protein level to reduce expression of its downstream targets, including HBV mRNA.

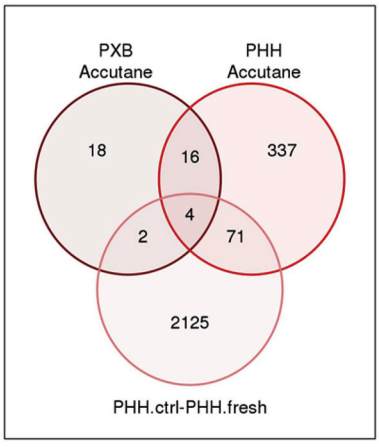
To characterize the pharmacodynamics effect of Accutane in our PXB mouse model, we conducted a full transcriptome analysis comparing liver tissues of treated PXB mice with those from the vehicle group.

As the PXB mice in our Accutane study were clearly at the edge of tolerability, implying a clear pharmacodynamic effect, we were surprised to find that the human gene changes in the liver transcriptome were vastly muted compared to the in vitro situation (Fig. 3). Not only are there an order of magnitude fewer genes affected (40 and 11 genes over 4-fold up- or downregulated, respectively), but the overlap between PHH and PXB liver tissue is also very poor (only 20 upregulated and 2 downregulated genes are shared between PHH and PXB liver sets). While it is clear that Accutane engaged some of its targets, e.g. CYP26A1 and CYP26B1 are upregulated 9.3- and 5.9-fold, respectively, and CYP7A1 is downregulated 2.9-fold (see Table S1 for GSEA scores), we find no significant changes for SHP and theHNF4α-related target genes. One possible explanation for the lack of efficacy in our PXB mouse model is that Accutane's in vivo effects on the transcriptome are simply less powerful than in vitro. However, it is important to realize that our transcriptomics analysis compares the difference of treatment effect from two distinct systems. The baseline for PHH is the DMSO control while in PXB mice it is the vehicle treated group. Since fresh hepatocytes must receive specific treatment (see Methods) to persist in cell culture and become permissible to HBV infection, this process should be taken into account when comparing tissue derived RNAseq data with cultured hepatocyte derived data. To this end we characterized transcriptome changes that occur when fresh PHH are cultured (Ichihara et al., 1982; Laishes and Williams, 1976).

Indeed, this process leads to more numerous transcriptome changes compared to Accutane treatment of cultured PHH. Fig. 3 shows the Venn diagram for genes up- or down-regulated by more than 4-fold for the three RNAseq datasets. Many of the typical retinoid target genes are strongly affected by the culturing process. Fig. 4a shows the effect on the 109 retinoid target gene set of the gene ontology database (GO) (Lomax, 2005; Osborne et al., 2007). The overall trend indicates that the response to Accutane in vitro and in vivo is quite different than what is seen upon culturing hepatocytes. However, some of the most retinoid responsive genes mentioned above, i.e. CYP26A1, CYP26B1, RARα and RARβ, clearly show that they are affected by culturing as well, but in the opposite direction (Fig. 4b). Regarding the SHP/HNF4α hypothesis for mechanism of antiviral activity, the data showed that PHH culturing strongly downregulates SHP, while retinoid treatment reverses that effect. If we assume that PXB mouse liver should be more similar to fresh hepatocytes, it seems that Accutane treatment of cultured PHH simply reverses some of the transcriptional changes induced by the culturing process. That is consistent with the reduced effect of Accutane in vivo, as we observed in the PXB study.

4. Discussion

Our quest for epigenetic and transcriptional regulators of HBV replication led us to the class of RAR agonists, represented by natural ligands ATRA and Accutane, as well as clinical, synthetic retinoids Acitretin and Tamibarotene. Note that a hint toward this connection was described as early as 1993 (Hsu et al., 1993) when 10 μM ATRA was shown to modulate HBsAg expression in a biphasic manner. However, this finding was not followed up until recently. Parallel to our discovery, a recent paper details potent anti-HBV activity for ATRA, Acitretin and Tamibarotene in HepRG-NTCP cells (Nkongolo et al., 2019). However, unraveling how these retinoids negatively affect HBV replication is a formidable task considering that regulation of hundreds of genes is ATRA-dependent. An ambitious 2002 analysis of 1192 papers covering 532 retinoid dependent genes attempts to classify these genes (Balmer and Blomhoff, 2002). While only 27 genes were deemed unquestionable direct targets, another 103 were likely candidates. This analysis also showed that retinoid effects are highly context dependent, i.e., cell type and time of treatment. A more recent study (Delacroix



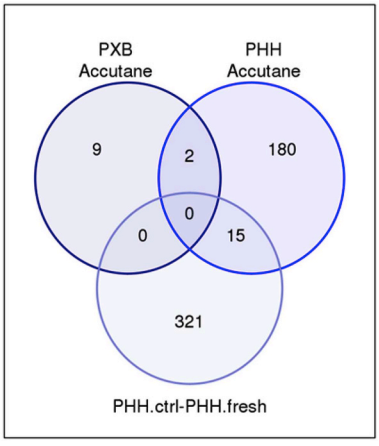


Fig. 3. RNAseq analysis comparing three experimental systems: (PXB Accutane) in vivo PXB mouse liver gene changes of Accutane treatment versus vehicle control, (PHH Accutane) in vitro PHH treated with 50uM Accutane for 32h versus DMSO control, (PHH.ctrl-PHH.fresh) fresh PHH versus cultured for 3 days) 4 < upregulated (left) 4 < downregulated (right).

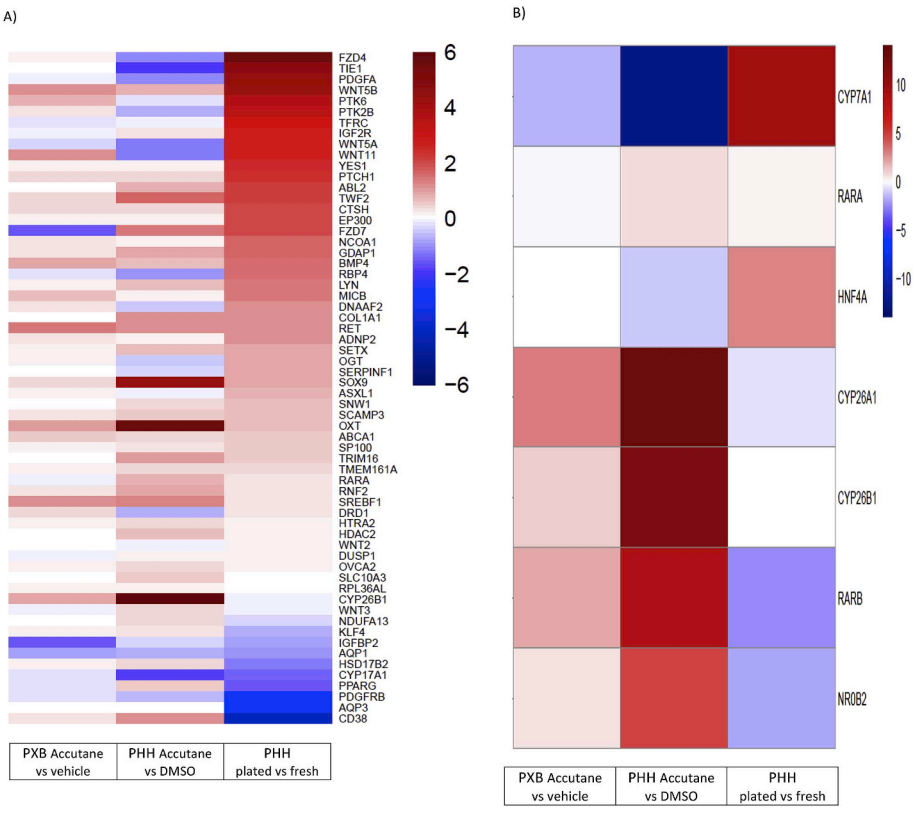


Fig. 4. RNAseq response for specific genes responsive to retinoids and implicated in HBV replication. (A) 109 ATRA responsive genes from GO for comparing cultured versus fresh hepatocytes (right), Accutane treated cultured hepatocytes versus DMSO (mid) and liver tissue from treated versus untreated PXB mice from our HBV efficacy study (left). (B) Selected, highly retinoid responsive genes in comparison to HBV transcription factor HNF4α and its repressor SHP (NR0B2).

et al., 2010) of differential RAR dependent gene expression in mouse embryonic fibroblasts versus embryonic stem cells illustrates remarkable cell-type dependent and temporal differences. It is also demonstrated that RAR-target genes cannot be upregulated by ATRA just under any condition, presumably, because expression is high already. In light of this unclear mechansim we focused on the translational question whether retinoids' in vitro activity could potentially have clinical utility.

Assuming that the retinoid antiviral effect is due to on-target activity, it is unlikely that typical lead optimization of any of the four candidates could arrive at a more potent compound with improved toxicity profile. Note that synthetic Tamibarotene is equipotent with ATRA for RARα and one or two orders of magnitude less potent for RARβ and RARγ, respectively (Lefebvre et al., 1995), but it does not have a higher tolerability and its clinical path is still uncertain. Our choice of a tool compound for the PXB mouse model was driven less by absolute potency but rather the potential of high exposure (several fold above EC_{50}) and the ease of a subsequent clinical evaluation. While Accutane is the least potent of the four drugs, its superior solubility in dosing vehicle, essential for high exposures, and its widespread clinical use made it an easy choice. After finding the optimal dosing vehicle in Balb/c mice, we established that the highest achievable dose was also well tolerated (a 0.6-fold adjustment was necessary for PXB mice). While the PXB mouse model with humanized liver might not be the best animal model to for all potential HBV-antivirals due to its altered bile acid metabolism (Chow et al., 2017; Naugler et al., 2015) our choice was driven by the expectation of more human-like metabolism (Bateman et al., 2014; De Serres et al., 2011; Kitamura and Sugihara, 2014; Klumpp et al., 2018; Mueller et al., 2018; Uchida et al., 2017). In humans but not in Balb/c mice, Accutane exhibits a major metabolite, 4-oxo-accutane, compound **19**, that accumulates to levels well above its parent (Colburn et al., 1983). Since this metabolite also has anti-HBV activity we chose the "more human" PXB system. To our disappointment, the metabolic profile for Accutane in PXB mice remained very similar to that of Balb/c mice and only insignificant amounts of the human metabolite were observed.), During the 4 week HBV efficacy study considerable weight loss occurred, but even with approaching the tolerability limit, there was no hint of antiviral efficacy.

A simple explanation of our negative in vivo study could be that the achieved pharmacological exposure was simply not sufficient, a hypothesis that is difficult to test due to Accutane's dose-limiting toxicity. It is also possible that the particular retinoid-dependent mechanism cannot be tested in this model due to the disrupted "murine gut to human liver" signaling (Chow et al., 2017) although we found several ATRA-dependent genes engaged in our PXB study. Nevertheless, it seemed prudent to investigate the in vitro/in vivo disconnect seen in an animal model that is clearly responsive to a number of established anti-HBV agents, such as PegInterferon and Entecavir (Uchida et al., 2017) as well as emerging ones, such as a HBV Capsid inhibitor (Klumpp et al., 2018) and a novel inhibitor of HBV mRNA processing (Mueller et al., 2018). Our transcriptome analysis revealed striking differences in gene expression when comparing Accutane treated hepatocytes with those from liver samples. Transcriptional changes induced by Accutane in PXB liver were vastly muted compared to the PHH system, pointing at the importance of the context dependence of retinoid action described above. In addition, the dysrgegulation of some metabolic pathway in the humanized PXB liver complicates the interpretation as the FXR/SHP axis has been described as downregulated compared to regular mouse liver (Naugler et al., 2015). However, since our transcriptomics analysis compares the differences between two systems that cannot be standardized to the same baseline, we characterized the transcriptional changes that occur when the in vivo system is manipulated into the in vitro cell culture, the process of turning liver biopsies into cultured hepatocytes. We were not able to utilize the PXB mouse liver samples, but we compared expression profiles of fresh PHH with those from 3 day cultured ones and found a large number of changes, many of which included ATRA-dependent genes such as SHP, a negative regulator of transcription factor HNF4α.

Clearly, the difference between a hepatocyte in its natural tissue context and a plated hepatocyte should be far larger than what we

capture by conditioning commercial hepatocytes toward being stably infected with HBV as described in Methods section. But our transcriptome analysis between these two latter conditions demonstrates profound changes for hundreds of genes. Many of these genes change in the reverse direction when exposed to retinoids, thereby potentially undoing key aspects of the culturing process.

We noted that a key feature of this process is the continuous treatment with glucocorticoid receptor agonist dexamethasone. This agent has been an essential media component since the early seventies (Ichihara et al., 1982; Jeejeebhoy and Phillips, 1976; Laishes and Williams, 1976) when it was shown to increase longevity of hepatocytes, maintain their biochemical liver functions and keeps them from de-differentiating (Favre et al., 2003). Clearly, the cellular effects of dexamethasone treatment are complex and much work has been directed to it (Adcock, 2001; Payne and Adcock, 2001; Thompson, 1987). While elucidating the interplay between dexamethasone and retinoids on the transcriptional level is well beyond the scope of this study, there is at least one example for the antagonizing effect for these transcription factors. Nuka et al. describe a human osteoblastic cell culture system in which dexamethasone treatment leads to cell mineralization via activation of alkaline phosphatase. This process was effectively inhibited by ATRA and Tamibarotene (Nuka et al., 1997).

Regardless of the potential issue of the PXB model not being ideal for every possible mechanism of action, the oppositional gene expression changes observed for the different states of our PHH assay system make room for the possibility of compounds showing artificial activity. When combining all of our observation, including the lack of retinoid activity in the HepG2-NTCP system and the three HBV patients, we believe that retinoid anti-HBV activity in PHH is artificial in that retinoids may counteract some of the changes associated with the culturing process, facilitated by dexamethasone. Note that the HepaRG-hNTCP assay for which retinoid anti-HBV activity was described earlier also requires dexamethasone treatment (Nkongolo et al., 2019). It is thus conceivable that the transcriptional status of the hepatocytes in PXB liver, and by extension human liver, cannot be affected by retinoids in the same way, leading to a translational disconnect for retinoids' anti-HBV activity. While our retinoid case can only be seen as an illustration of the potential problem with the dexamethasone dependent PHH system, it prompted us to be more cautious with novel chemotypes emerging from PHH phenotypic screening especially when they are not active in any other celltype permissible to studying HBV replication.

5. Conclusion

It is generally assumed that phenotypical screening is most powerful when the cellular assays system is as close as possible to the pathological in vivo situation. Earlier disconnects between PHH and HepG2-NTCP assay systems, i.e. established HBV drug IFN is only effective in PHH, led us to use the latter as the more relevant system. Approved retinoid drugs showed anti-HBV activity in PHH but failed to do so in HepG2-NTCP cells. The lack of Accutane's activity in the PXB mouse model of HBV infection, despite higher exposures than used in the clinic, suggests that the PHH system may lead to potentially false positives. Of course, we cannot rule out that the PXB mouse model might not reflect activity from all cellular mechanism due to its altered bile acid metabolism. However, our PHH transcriptome analysis showed that retinoids increase SHP levels decreasing HNF4α dependent transcription, which has been described before. As the dexamethasone dependent culturing process leads to downregulation of SHP, it is possible that retinoids simply reverse some of the conditions required for stable HBV infection. This could lead to an artificial antiviral effect that would not be expected to translate in the PXB model. Clearly, other hypothesis could be found and pursued, but taken all of our observations together, there is not enough evidence to test retinoids in the clinic.

Conflicts of interest

Yoshida Morikawa is an employ of Phoenix Bio. All other authors were employees of Gilead Sciences when the work was conducted. All authors declare no competing interests.

Acknowledgements

We thank Hassan Javanbakht and Guofeng Cheng from the Gilead HBV biology group for help with many details of the work and the manuscript. We are grateful to Frank Chisari for his many helpful comments and suggestions during the course of the studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104538.

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