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Chapter 7

Identification of FDA-approved drugs that target hepatitis B virus transcription

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Abstract

In the treatment of chronic hepatitis B virus (HBV) infection, polymerase inhibitors successfully suppress HBV DNA production. However, the production of viral proteins continues unhindered, which hampers viral clearance. Here, we screen for compounds that suppress HBV transcription, which would prevent viral protein production. 640 FDA-approved drugs were evaluated for their ability to inhibit HBV transcription in a transfection-based HBV reporter assay. The assay was performed in the presence and absence of the HBV accessory protein X (HBx), which is essential for in vivo HBV RNA transcription. We observed that in the absence of HBx 47, and in the presence of HBx 24 compounds suppressed transcription by more than 20%. We selected the 24 most potent compounds in each condition for further analysis. On average, the selected compounds reduced transcription by 33.9% (Range: 24.1%-65.8%) in the absence of HBx expression, and 30.6% (Range: 20.4%-48.9%) in the presence of HBx. The two selections of 24 compounds had 12 compounds in common, resulting in a final selection of 36 compounds, which were evaluated for their capacity to suppress HBV replication in constitutively HBV replicating HepG2.2.15 cells. Twenty-three of these compounds reduced HBV replication by interfering with RNA transcription. Further analysis revealed that one of the compounds, terbinafine, potently and specifically suppressed HBx-mediated HBV RNA transcription in HepG2 cells. Inhibition of HBV protein production is a promising step towards HBV clearance. In combination with an HBV polymerase inhibitor, the added suppression of HBV RNA transcription may markedly improve antiviral treatment outcome.
**Introduction**

After acute infection with hepatitis B virus (HBV), most adults clear the infection, while the majority of children develop chronic HBV infection (CHB). Although HBV vaccination has greatly reduced HBV incidence in many areas, worldwide the number of people with CHB still increased from an estimated 223 million people in 1990 to 240 million in 2005\(^5\). Consequently, CHB is still a major healthcare problem that is responsible for more than 780,000 deaths annually.

HBV infects human hepatocytes. After cellular entry, the viral DNA genome is released into the nucleus where it is processed by host proteins into fully double stranded covalently closed circular DNA (cccDNA). HBV pregenomic RNA (pgRNA) is packaged into core particles together with the viral polymerase, which reversely transcribes the pgRNA into a partially double stranded viral DNA genome. The core particles are enveloped and secreted, but can also be shuttled back to the nucleus to increase the cccDNA pool. Beside mature virus, HBV infected hepatocytes excrete large amounts of the viral surface protein (HBsAg), and often also of the HBV e-antigen (HBeAg). The presence of these antigens has been linked to failure to develop a successful immune response\(^2,3\). In natural infection, partial immunological control of HBV replication correlates with the disappearance of HBeAg, and a reduction of serum HBV DNA levels. At a very low rate, concomitant with the appearance of functional HBV-specific CD8+ T cells, CHB patients clear all markers of viral replication\(^4\).

Currently the therapeutic options for CHB are long-term treatment with the nucleotide analogues entecavir or tenofovir; or a course of pegylated interferon α (PegIFNα)\(^5,8\). Nucleotide analogues block the conversion of the pgRNA into DNA, preventing the production of infectious mature virus and the production of new cccDNA. Nucleotide analogues do not affect the production of viral antigens. PegIFNα activates innate immune pathways that interfere with viral replication. In some experimental systems interferon partially suppresses HBV transcription\(^9,10\), but in CHB patients PegIFNα treatment does not prevent viral protein production.

HBV encodes one accessory protein called X (HBx) that is essential for HBV replication in vivo. In the absence of HBx, the HBV cccDNA is epigenetically inactivated, eventually completely preventing HBV RNA transcription. It is unknown how HBx affects HBV transcription. In animal models, HBx-deficient HBV replicates poorly\(^11\) or not at all\(^12-14\), and shRNA-mediated knockdown of HBx can potently suppress viral replication even after HBV infection is established\(^15\).

Here, we investigate whether the pathways modulated by HBx are targets for therapeutic intervention. We use a semi high-throughput, transfection-based in vitro HBV transcription assay to screen the 640 compounds in ENZO’s FDA approved drug library for compounds that suppress HBV transcription. The screen identified 36 HBV transcription inhibitors, of which the majority indeed suppressed HBV replication in hepatoma derived HepG2.2.15...
cells. One HBV transcription inhibitor specifically suppressed HBx mediated transcriptional transactivation, suggesting functional interference. Application of the compounds identified in this study for treatment of CHB may improve antiviral therapy outcome by specific interference with the viral protein production, which is not affected by HBV polymerase inhibitors.

Materials and Methods

Cell culture and transfection
HEK 293 cells were maintained in Dulbecco’s Modified Eagle Medium without HEPES (DMEM) (LONZA, Basel, Switzerland) supplemented with 10% heat inactivated fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) (Gibco Pen Strep) in a humidified 5% CO2 incubator at 37°C. HepG2 and HepG2.2.15 cells were maintained in William’s Medium E w/o L-Gln (LONZA, Basel, Switzerland), supplemented with 10% v/v inactivated fetal calf serum, 2 mM L-glutamin (LONZA, Basel, Switzerland), penicillin (100 U/mL), streptomycin (100 µg/mL) and 5 µM Dexamethasone (Sigma Aldrich). HepG2 cells were maintained in a humidified 10% CO2 incubator at 37°C, and HepG2.2.15 cells were maintained in a humidified 5% CO2 incubator at 37°C. Twenty-four hours before transfection the cells were plated into 96-well (HEK 293), 24 well (HepG2.2.15) or 6-well (HepG2) culture plates. The calcium phosphate method was used for transfection. Briefly, plasmid DNA was diluted in 42 mM HEPES pH 7.2 and 2.5 M CaCl2 was added to a final concentration of 0.15 M CaCl2. The DNA mixture was added to an equal volume of 2x HEPES buffered saline (HBS)(275 mM NaCl, 10 mM KCl, 1.4 mM Na2HPO4, 42 mM HEPES pH 7.2) and after a 15 minute incubation at room temperature the mixture was added to the cells. Cells were incubated overnight in a humidified 3% CO2 incubator at 37°C and subsequently the medium was replaced and compounds were added. The amount of DNA transfected per well was kept constant by cotransfection of our vector control.

Expression vectors
The pHSV-HBx vector expressing HSV-tagged HBx was previously generated and was previously described. The R9 vector, which contains a 1.2 x overlength HBV DNA genome (subtype adw) in a pGEM 7zf+ backbone, was kindly provided by Dr. Baumert. To create an R9 vector lacking HBx expression (R9ΔX), the Glu97→STOP and Met103→Arg mutations were introduced by site directed mutagenesis as described previously. The primers to generate the luciferase reporters under control of the HBV core promoter were adapted from Du et al. to make them suitable to the HBV adw subtype and were as follows: Corepromoter-F: 5’-CCCGAGCTCAAAGTCTTACAAG-3’; Corepromoter-R: 5’-CCCAAGCTTTGGAGGCCTGAACAGT-3’. Using these primers, the HBV core promoter was amplified by PCR from the R9 vector. The PCR product containing the HBV core
promoter was cut with SacI and HindIII and ligated in front of the luciferase reporter of the pGL3Basic vector (Promega). As a vector control, the empty pcDNA 3.1 A(-) (Invitrogen) was used. All constructs were validated by BDT sequencing.

**Compounds and screening assay**

The 640 compounds in the Screen-Well FDA approved Drug Library (BML-2841 v.1.5, Enzo Life Sciences) were screened for their effect on HBV transcription. The compounds were supplied in 96 well plates, each plate containing 80 compounds at a concentration of 2 mg/ml in DMSO. For each plate that was screened, one 96 well plate was seeded with HEK 293 cells and after 24 hours transfected with pCore-Luc alone or pCore-Luc and pHSV-HBx and subsequently treated with compounds at a final concentration of 20 µg/ml and 1% DMSO. The remaining 16 wells were also transfected and treated with vehicle (DMSO) to serve as controls. To assess the relative effect of compounds on luciferase transcription, luciferase activity was normalized as a percentage of the row average. For further characterization, terbinafine and physostigmine sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Cell viability**

Cell viability was assessed by MTT assay. Thiazolyl Blue Tetrazolium Blue (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). After 24 hour incubation with compounds, MTT was added to a final concentration of 0.5 mg/ml and cells were incubated for another 3 hours in a humidified 5% CO2 incubator at 37°C. Next, medium was aspirated, 100 µl DMSO was added and absorbance was read out at 580 nm. Background was determined by measuring absorption at 655 nm and subtracted from the measurement at 580 nm. The effect of compounds on HepG2 and HepG2.2.15 cell viability was assessed by microscopy. After 6 days of culture in absence of compounds, confluence reached 95-100%. When in presence of a compound confluence was below 80%, that compound was excluded (indicated in Table 1). Compounds that reduced confluence to below 95% were designated as causing mild (cellular growth inhibition 0-15%) and are indicated Table 1.

**Luciferase assay**

Luciferase activity was measured 24 hours after adding compounds by adding 25 µl substrate (0.83 mM ATP, 0.83 mM D-luciferin (Duchefa, Haarlem, The Netherlands), 18.7 mM MgCl2, 0.78 µM Na2H2P2O7, 38.9 mM Tris pH 7.8, 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 µM dithiothreitol) directly to the culture medium. Luminescence was measured for 1 s per well using a luminometer (Berthold, Bad Wildbad, Germany).
Quantification of HBV RNA transcription and virus production

HepG2.2.15 cells were seeded in 24 well plates in the presence of candidate compounds at a final concentration of 20 µg/ml compound and 1% DMSO. Control wells were treated with DMSO only. After three days of culture, medium was refreshed and supplemented with fresh compounds. HepG2 cells were transfected with the R9 vector or its mutant R9ΔX to initiate HBV replication in the presence and absence of HBx, respectively. After transfection, medium was changed and compounds were added at the indicated concentrations. DMSO was added to a final concentration of 2% under all conditions. After three days of culture, medium was refreshed and supplemented with fresh compounds. To assess virus production, encapsidated HBV DNA in the supernatant of HBV replicating cells was quantified by qPCR as described previously. To assess HBV RNA production, total RNA of HBV replicating cells was purified using TriPure Isolation Reagent (Roche) according to the manufacturer’s instructions. To degrade remaining DNA contamination, the RNA was treated with RQ1 RNAse free DNAse (Promega) and cleaned up using the NucleoSpin® RNA Clean-up kit (Macherey-Nagel). cDNA was prepared using random hexamers (Invitrogen) and 200 U M-MLV reverse transcriptase (Promega, Madison, WI, USA) in the presence of 20 U RNAsin (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Subsequently, 2 µl of cDNA was used in a quantitative PCR using the SYBR Green I Master (Roche) and a LightCycler® 480 system (Roche). HBV cDNA was quantified relative to β-actin mRNA cDNA. The qPCR primers used to amplify HBV cDNA were previously described and the primers to quantify β-actin mRNA cDNA were BA-F: 5′-GGCCAGTCCTCTCCCAAGTCCAC-3′ and BA-R: 5′-GGTAAGCCCTGGCTG-CCTCCACC-3′. The following program was used for qPCR: 10 min 95°C, followed by 50 cycles of 10 sec 95°C, 20 sec 59°C, 30 sec 72°C with a single acquisition during the 72°C step.

Data analysis and statistics

The heatmap that shows the effect of the different compounds on HBV replication in our assay was generated using the HeatMapImage module from GenePattern. The measurements of HBV replication in HepG2 and HepG2.2.15 were analyzed using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA). HBx specificity of compounds inhibiting HBV RNA production by >50% was determined by a one-sided t test. Significance of differences in luciferase activity and HBV DNA production were all determined by two-sided t tests. The IC50 and IC90 for terbinafine were calculated from using linear regression analysis using GraphPad Prism 5.01.
**Results**

Identification of compounds that suppress HBV transcription using a luciferase-based, semi high throughput assay

The 640 compounds in ENZO’s FDA-approved drug library were screened for suppression of HBV transcription. Before assessing their effect on transcription, cytotoxic compounds were excluded from further analysis. Cytotoxicity of the compounds was analyzed using the MTT assay on HEK 293 cells that were exposed to the compounds at a final concentration of 20 µg/ml for 24 hours. In total 360 of the 640 compounds reduced MTT activity by more than 10%, and were excluded from further analysis (Figure 1, Table S1).

To screen for compounds that suppress HBV transcription in a semi high throughput manner, we generated a DNA construct from which luciferase is transcribed under control of the HBV core promoter, pCore-Luc. When HEK 293 cells were transfected with this construct, we observed that HBx expressed from a cotransfected construct (pHSV-HBx) transactivated luciferase transcription, indicating that the regulatory mechanisms modulated by HBx are present in these cells. To screen for HBV transcription inhibitors among the 280 selected non-toxic compounds, HEK 293 cells were transfected with pCore-Luc and cotransfected with pHSV-HBx or empty vector DNA (EV)(Figure 1). Subsequently, transfected cells were treated for 24 hours with the different compounds at a final concentration of 20 µg/ml after which luciferase activity was assessed. Based on three independent experiments, compounds were ranked on their capacity to reduce transcription in the absence (Table S2) and presence (Table S3) of HBx, respectively.

![Figure 1. Schematic overview of library screen workflow](image-url)
HBx expression stimulated transcription by ~20% in our assay, we therefore consider 20% inhibition as a cutoff for compound activity. We observed 24 compounds which suppressed transcription by more than 20% in the presence of HBx, and 47 compounds which suppressed transcription by more than 20% in the absence of HBx. The 24 compounds suppressing HBV replication in the presence of HBx, as well as the 24 most potent inhibitors in the absence of HBx, were selected for further analysis. The two selections of 24 compounds had 12 compounds in common, resulting in a final selection of 36 compounds. On average, the selected compounds reduced transcription by 33.9% (Range: 24.1%-65.8%) in the absence of HBx expression, and 30.6% (Range: 20.4%-48.9%) in the presence of HBx. Two of the 12 compounds suppressing transcription in the presence of HBx (dexamethasone and nimesulide), also suppressed in the absence of HBx but were not in the top 24; resulting in the 12-14-10 distribution as summarized in Fig. 2A and 2B.

Figure 2. Overview of compounds that suppress HBV transcription. (A) Heatmap of the 24 most potent inhibitors of HBV transcription in the presence and absence of HBx, respectively. Values represent transcriptional activity of a luciferase reporter that represents the HBV cccDNA after 24 hours of treatment with the indicated compounds from ENZO’s FDA approved drug library. (B) HBx-dependency of compounds suppressing transcription by more than 20%. 14 compounds suppressed HBV transcription in our assay in the presence and absence of HBx.
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(A) Heatmap of the 24 most potent inhibitors of HBV transcription in the presence and absence of HBx, respectively. Values represent transcriptional activity of a luciferase reporter that represents the HBV cccDNA after 24 hours of treatment with the indicated compounds from ENZO’s FDA approved drug library. (B) HBx-dependency of compounds suppressing transcription by more than 20%. 14 compounds suppressed HBV transcription in our assay in the presence and absence of HBx.

Table 1. Effect of HBV transcription inhibitors on HBV replication in HepG2.2.15 cells

<table>
<thead>
<tr>
<th>Compound name</th>
<th>HBV DNA production (%)</th>
<th>HBV RNA transcription (%)</th>
<th>Cellular growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lomerizine</td>
<td>72,96</td>
<td>7,95</td>
<td>-</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>46,07</td>
<td>16,63</td>
<td>-</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>49,79</td>
<td>16,75</td>
<td>-</td>
</tr>
<tr>
<td>Bromocriptine mesylate</td>
<td>46,01</td>
<td>18,78</td>
<td>-</td>
</tr>
<tr>
<td>Methyldopa</td>
<td>38,70</td>
<td>21,49</td>
<td>-</td>
</tr>
<tr>
<td>Physostigmine sulfate</td>
<td>140,72</td>
<td>29,70</td>
<td>-</td>
</tr>
<tr>
<td>Trequinsin·HCl</td>
<td>38,55</td>
<td>31,41</td>
<td>-</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>71,49</td>
<td>50,76</td>
<td>-</td>
</tr>
<tr>
<td>Fenoldopam-HCl</td>
<td>70,41</td>
<td>53,08</td>
<td>-</td>
</tr>
<tr>
<td>Finasteride</td>
<td>96,13</td>
<td>73,33</td>
<td>-</td>
</tr>
<tr>
<td>Levodopa</td>
<td>43,70</td>
<td>81,99</td>
<td>-</td>
</tr>
<tr>
<td>DL-Isoproterenol</td>
<td>84,74</td>
<td>83,10</td>
<td>-</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>68,05</td>
<td>83,37</td>
<td>-</td>
</tr>
<tr>
<td>Abaramectin</td>
<td>71,20</td>
<td>87,48</td>
<td>-</td>
</tr>
<tr>
<td>Etidronate</td>
<td>75,97</td>
<td>91,73</td>
<td>-</td>
</tr>
<tr>
<td>Glipizide</td>
<td>81,66</td>
<td>103,10</td>
<td>-</td>
</tr>
<tr>
<td>Disodium Cromoglycate</td>
<td>111,64</td>
<td>120,12</td>
<td>-</td>
</tr>
<tr>
<td>Benserazide</td>
<td>75,01</td>
<td>121,30</td>
<td>-</td>
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<tr>
<td>Tranilast</td>
<td>76,99</td>
<td>124,99</td>
<td>-</td>
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<tr>
<td>Dexamethasone</td>
<td>96,13</td>
<td>140,49</td>
<td>-</td>
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<tr>
<td>Clodronic acid</td>
<td>214,07</td>
<td>190,64</td>
<td>-</td>
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<td>Dipyridamole</td>
<td>272,75</td>
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<td>Melengestrol Acetate</td>
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<tr>
<td>Cilnidipine</td>
<td>21,91</td>
<td>34,14</td>
<td>0-15%</td>
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<tr>
<td>Nifedipine</td>
<td>24,25</td>
<td>66,03</td>
<td>0-15%</td>
</tr>
<tr>
<td>Calcipotriene</td>
<td>31,83</td>
<td>79,50</td>
<td>0-15%</td>
</tr>
<tr>
<td>Bexarotene</td>
<td>86,49</td>
<td>120,22</td>
<td>0-15%</td>
</tr>
<tr>
<td>Lapatinib ditosylate</td>
<td>N.D.</td>
<td>N.D.</td>
<td>15-50%</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>N.D.</td>
<td>N.D.</td>
<td>15-50%</td>
</tr>
<tr>
<td>Manidipine</td>
<td>N.D.</td>
<td>N.D.</td>
<td>15-50%</td>
</tr>
<tr>
<td>Tolmetin</td>
<td>N.D.</td>
<td>N.D.</td>
<td>15-50%</td>
</tr>
<tr>
<td>Loratadine</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Oxatomide</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Mifepristone</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Ethacrynic Acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>Vatalanib</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&gt;50%</td>
</tr>
</tbody>
</table>

HBV DNA production and HBV RNA transcription by HepG2.2.15 cells treated for 6 days with the indicated compounds at a concentration of 20 ug/ml in the presence of 1% DMSO. N.D.: Not determined.
Effect of HBV transcription inhibitors on HBV replication

Next, we assessed the effect of the 36 selected compounds on HBV replication. In HepG2.2.15 cells, HBV replication is initiated from integrated HBV DNA and as a result these cells constitutively replicate HBV. When HepG2.2.15 cells were exposed to our candidate compounds for 6 days, we observed that 9 compounds affected cell viability and these were excluded from further analysis (Table 1). Of the 27 remaining compounds, 23 suppressed the production of encapsidated HBV DNA in the supernatant and 19 suppressed HBV RNA transcription (Figure 3A and Table 1), albeit that some compounds had a minor effect on cell growth (Table 1). The HBV DNA production correlated well with the HBV RNA transcription, indicating that most compounds indeed suppressed HBV replication by interfering with HBV transcription (Figure 3B). However, some individual compounds differentially affected HBV DNA and HBV RNA transcription, which might indicate that these compounds can differentially affect the different HBV promoters and/or affect additional steps in HBV replication.

![Figure 3. Effect of HBV transcription inhibitors on HBV replication in HepG2.2.15 cells. (A) HepG2.2.15 cells, which constitutively replicate HBV, were treated with the compounds that suppressed HBV transcription in our screen. Compounds that affected HepG2.2.15 cell viability were excluded. Each dot represents the effect of a single compound. 23/27 compounds investigated suppressed the production of encapsidated HBV DNA in the supernatant and 19/27 suppressed HBV RNA transcription. (B) HBV RNA transcription correlated well with HBV DNA production. (C) Overview of compounds with an IC50 below 20 mg/ml for suppressing HBV RNA transcription and/or HBV virus production.](image)
We observed that 10 compounds suppressed HBV DNA production by more than 50% and 9 compounds suppressed HBV RNA transcription by more than 50%. 7 compounds suppressed both HBV RNA transcription and HBV DNA production (Figure 3C). The current clinical application and maximum duration of therapy for compounds with an antiviral effect is summarized in Table S4.

**Terbinafine suppresses HBV RNA transcription in an HBx-dependent manner and strongly reduces virus production**

In HepG2.2.15 cells HBx expression strongly augments HBV RNA transcription\(^{23,24}\). We observed that seven HBV compounds suppressed HBV RNA transcription in HepG2.2.15 cells by more than 50%, indicating that they may functionally interfere with HBx. Four of these compounds (trequinsin, terbinafine, physostigmine and capsaicin) suppressed HBV transcription in our transfection based assay by more than 20% in the presence, but not in the absence of HBx (Figure 1, Table 1). However, the difference in transcriptional inhibition in the presence and absence of HBx was only significant for terbinafine and physostigmine (Figure 1, Table 1) and for these compounds HBx specificity could be confirmed in a dose dependent manner (Figure 4A,B). Next, we investigated the effect of terbinafine and physostigmine on the full HBV replication cycle in the presence and absence of HBx. HepG2 cells were transfected with the R9 vector, which contains a 1.2x overlength copy of the HBV genome and initiates viral replication, and a mutant of this vector devoid of HBx expression, R9ΔX\(^{16,17}\). This mutant transcribed 78% less HBV RNA and produced 84% less HBV DNA in the supernatant.

When HBV replicating HepG2 cells were treated with physostigmine, we observed some HBx-specific inhibition of RNA transcription at the highest concentrations tested, but this trend was not significant (Supplementary figure 1A). Physostigmine did not significantly affect HBV virus production (Supplementary Figure 1B).

When HBV replicating HepG2 cells were treated with terbinafine for 6 days, we observed that terbinafine in a dose dependent manner suppressed HBV RNA transcription in the presence of HBx, but that terbinafine only had minor effect on HBV RNA transcription in the absence of HBx (Figure 4C). This suggests that terbinafine indeed counteracts the stimulation of HBV RNA transcription by HBx. In the presence of HBx, terbinafine suppressed HBV RNA transcription in HepG2 cells with 113.9 ± 14.14-1.413 ± 0.4563 %/µg/ml (half maximal inhibitory concentration (IC50): 40 µg/ml, 90% inhibiting concentration (IC90): 73 µg/ml). Terbinafine also strongly and dose dependently suppressed HBV virus production in HepG2 cells in the presence of HBx, with 93.93 ± 8.229-1.596 ± 0.2655 %/µg/ml (IC50: 29 µg/ml, IC90: 53 µg/ml). Surprisingly, terbinafine also suppressed HBV virus production in HepG2 cells in the absence of HBx expression, with 15.52 ± 1.365-0.3245 ± 0.04403 %/µg/ml (IC50: 24 µg/ml, IC90: 43 µg/ml) (Figure 4D). Terbinafine did not affect HepG2 viability at the highest concentration tested.
Figure 4. Terbinafine interferes with HBx-mediated transcription. (A,B) HEK 293 cells were transfected with a construct that represents the HBV cccDNA and cotransfected with a vector from which HBx is expressed. 24 hours of treatment with terbinafine or physostigmine suppressed RNA transcription specifically in the presence of HBx. (C) HepG2 cells were transfected with the R9 construct or the R9ΔX construct, which contain a 1.2x overlength copy of the HBV genome and initiate HBV replication in the presence and absence of HBx, respectively. Terbinafine suppressed HBV RNA transcription in HepG2 cells in a dose dependent manner in the presence of HBx only. (D) Terbinafine-mediated suppression of HBV RNA transcription strongly reduced HBV virus production in a dose dependent manner in the presence and even significantly reduced HBV virus production in absence of HBx.

Discussion

Chronic infection with hepatitis B virus is a major healthcare problem, and currently available therapeutic options are not curative. A major hurdle to clear HBV infection is the lack of effect of current therapy on the ongoing production of viral proteins. These proteins are produced from viral RNA that is transcribed from existing cccDNA in already infected hepatocytes. To overcome this problem, we investigated the possibility to inhibit HBV
transcription using compounds in ENZO’s FDA approved drug library, which typically can exert a biological effect in humans at tolerable concentrations.

We identified 47 compounds that suppressed HBV transcription by more than 20% in the absence of HBx expression, and 24 compounds that suppressed HBV transcription by more than 20% in the presence of HBx. We further investigated the most potent transcription inhibitors in HepG2.2.15 cells. The effect of the compounds on virus production correlated well with their effect on HBV RNA transcription, indicating that the compounds indeed suppressed transcription from the HBV cccDNA, confirming the validity of our transfection-based transcription assay. However, we cannot exclude some compounds affect other steps in the viral replication cycle and/or differentially affect the four HBV promoters. Such effects may be responsible for the outliers with a disturbed correlation between the effect on HBV RNA transcription and DNA production of some compounds.

The most potent inhibitor of HBV RNA transcription in HepG2.2.15 cells identified in our screen was lomerizine, an L-type calcium channel (LTCC) blocker, which reduced HBV RNA transcription by 92%. We identified two more L-type calcium channel blockers, cilnidipine and nifedipine, that also efficiently suppressed HBV replication in HepG2.2.15 cells. Interestingly, in our transfection-based assay LTCC blockers suppressed HBV transcription better in the absence- than in the presence of HBx, suggesting that in HEK 293 cells HBx can partially counteract the effect of these compounds. HBx expression induces the release of calcium from the mitochondria and the resulting increase in intracytosolic calcium is critical to its function. The expression and function of LTCC has not been investigated in hepatocytes, but in other cell types these transporters mediate extracellular calcium influx. Our results indicate that blocking LTCC in HEK 293 cells affects HBV transcription by decreasing intracellular calcium. HBx can partially counteract the effect of LTCC blockers by releasing calcium from the mitochondria. In HepG2.2.15 cells LTCC blockers potently suppressed HBV replication in the presence of HBx, suggesting that in these cells HBx cannot fully compensate for the decrease in intracellular calcium levels. Whether this mechanism can be therapeutically applied to suppress HBV replication in vivo will depend on the in vivo expression and function of L-type calcium channels in the liver; and on the occurrence of unwanted side effects. To assess their suitability for the treatment of CHB, the effects on HBV replication could be assessed in animal models of HBV replication, in the context of in vivo expression of L-type calcium channel expression. LTCC blockers are orally available, do not have severe side effects, and can be administered for more than a year.

Two of the compounds that efficiently suppressed HBV transcription in HepG2.2.15 cells, bromocriptine mesylate and levodopa, are dopamine receptor agonists. Dopaminergic signaling in vivo has profound effects on several aspects of hepatocyte function, and our results suggest that these effects effectively suppress HBV transcription and that they can be induced in hepatocytes using FDA approved drugs. Drugs that affect dopamine signaling are by nature psychoactive, and in general have severe side effects. However, these drugs are widely used, and possibly their effect on CHB can be derived from medical records in
countries where HBV is highly endemic. Dopamine receptor agonists are administered for years under treatment conditions for diseases such as Parkinson’s disease, suggesting that these compounds could be safely administered to complement the treatment for chronic HBV infection.47

Other HBV transcription inhibitors identified in our screen also potently suppressed HBV replication, but for these no common mechanism was evident (Table 2). It remains unclear whether all compounds we identified could reach sufficiently high concentrations in the liver to effectively suppress HBV replication in chronically infected patients. However, all compounds we investigated have been approved by the FDA because they can achieve a biological effect in humans at tolerable concentrations.

HBV replication in vivo critically depends on HBx expression.12,14 The capacity to transactivate RNA transcription in vitro is related to the essential role of HBx in vivo.30 Two of the compounds that efficiently suppressed HBV RNA transcription in HepG2.2.15 cells, terbinafine and physostigmine, specifically suppressed HBV transcription in our transfection based assay in the presence of HBx. Therefore we investigated whether these compounds functionally counteract HBx. In HepG2 cells, physostigmine did not reduce HBV RNA transcription in a dose dependent manner, although in the presence of HBx some inhibition was observed at the highest concentrations we tested. Nevertheless, physostigmine did not suppress HBV virus production in HepG2 cells and therefore is not suitable for treatment of CHB and we did not investigate this compound further.

For terbinafine our results indicate that also in HepG2 cells this compound specifically counteracts transcriptional transactivation by HBx. Terbinafine potently suppressed HBV RNA transcription in a dose-dependent manner in the presence of HBx, but it only had a minor, not significant effect on HBV RNA transcription in the absence of HBx. The reduction of HBV RNA transcription strongly affected HBV virus production, which was reduced stronger than HBV RNA transcription was reduced by equal amounts of terbinafine. Even the minor effect of terbinafine on HBV RNA transcription in the absence of HBx significantly affected HBV virus production.

Terbinafine (SF 86-327, Lamisil) is an allylamine derivate that was originally identified as an inhibitor of fungal squalene epoxidase.31,32 In addition it interferes with various pathways in eukaryotic cells, affecting processes such as the cell cycle, viability and apoptosis.33 Terbinafine can be safely administered to patients for more than a year without severe side effects. The antiviral effect of terbinafine is not related to the canonical clinical function, inhibiting squalene epoxidase. The peak plasma concentration of terbinafine after four weeks of administration of the most commonly used clinical dose, 250mg, is 1.70 ± 0.77 µg/ml.34 This is below the IC50 of the antiviral effect of terbinafine observed in HepG2.2.15 and HepG2 cells. However, higher dosages, up to 1000 mg/day have also been reported to be safe for prolonged use.35 Terbinafine is highly hydrophobic and the concentration widely differs between different tissues.36 After intravenous injection, terbinafine predominantly distributes
to the liver, suggesting that the concentrations in this organ might be higher than the peak plasma concentration and may reach levels sufficient to interfere with HBV replication. Like other antifungal agents, terbinafine can cause severe acute liver injury in patients with and without a preexisting liver condition. These exacerbations are rare, even in highly HBV endemic countries, such as Taiwan, where terbinafine is prescribed without prior HBV infection.

### Table 2: Mechanism of action of compounds with an IC50 below 20uM for suppressing HBV RNA transcription and/or HBV virus production.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Suppression of HBV replication (% control)</th>
<th>Mechanism of Action:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transcription * No HBx + HBx DNA** RNA***</td>
<td></td>
</tr>
<tr>
<td>Lomerizine</td>
<td>31% 19% 27% 92%</td>
<td>LTCC and TTCC blocker</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>0% 22% 54% 83%</td>
<td>Squamous epoxidase inhibitor ****</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>5% 20% 50% 83%</td>
<td>Vanilloid receptor subtype 1 activator</td>
</tr>
<tr>
<td>Melengestrol Acetate</td>
<td>34% 2% 57% 82%</td>
<td>steroidal progestin</td>
</tr>
<tr>
<td>Bromocriptine mesylate</td>
<td>39% 22% 54% 81%</td>
<td>Dopamine receptor agonist</td>
</tr>
<tr>
<td>Methyldopa</td>
<td>30% 8% 61% 79%</td>
<td>α-adrenergic receptor agonist</td>
</tr>
<tr>
<td>Physostigmine sulfate</td>
<td>8% 20% -41% 70%</td>
<td>Cholinesterase inhibitor</td>
</tr>
<tr>
<td>Trequinsin-HCl</td>
<td>19% 25% 61% 69%</td>
<td>PDE3 inhibitor</td>
</tr>
<tr>
<td>Cilnidipine</td>
<td>31% 13% 78% 66%</td>
<td>LTCC and NTCC blocker</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>35% 15% 76% 34%</td>
<td>LTCC blocker</td>
</tr>
<tr>
<td>Calcipotriene (Calcipotriol)</td>
<td>51% 34% 68% 20%</td>
<td>Vitamin D-like</td>
</tr>
<tr>
<td>Levodopa</td>
<td>35% 13% 56% 18%</td>
<td>Dopamine receptor agonist</td>
</tr>
</tbody>
</table>

*: Percentage suppression of HBV transcription in the absence and presence of HBx. HBx specific compounds are indicated in bold; **: Percentage suppression of encapsidated HBV DNA production by HepG2.2.15 cells relative to control; ***: Percentage suppression of HBV RNA transcription in HepG2.2.15 cells relative to control; ****: The effect of Terbinafine is not related to its capacity to inhibit squamous epoxidase, as this protein is not expressed by eukaryotic cells. Terbinafine affects various processes in eukaryotic cells. Compounds are ranked by their capacity to suppress HBV RNA transcription in HepG2.2.15 cells. LTCC: L-type calcium channel; TTCC: T-type calcium channel; NTCC: N-type calcium channel; PDE3: Phosphodiesterase 3.
Nevertheless, liver damage, as indicated by an increased ALT level, is a contraindication for terbinafine use. Therefore terbinafine should only be studied in CHB patients already on HBV polymerase inhibitor maintenance therapy, in whom ALT is normalized.

There is a need for drugs that interfere with the ongoing production of HBV proteins in CHB patients, in whom HBV DNA is suppressed by HBV polymerase inhibitor maintenance therapy. Using an innovative screening approach, we identified two classes of FDA-approved drugs, and several individual FDA-approved drugs that potently suppressed HBV RNA transcription \textit{in vitro}. Application of these drugs in chronic hepatitis B patients, together with an HBV polymerase inhibitor, may substantially reduce viral protein production, facilitating clearance of the infection.
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References


Supplementary information:

Supplementary figure 1. Effect of physostigmine on HBV replication in HepG2 cells. HepG2 cells were transfected with the R9 construct or the R9dX construct, which contain a 1.2x overlength copy of the HBV genome and initiate HBV replication in the presence and absence of HBx, respectively. (A) High concentrations of physostigmine suppressed HBV RNA transcription in HepG2 cells in a dose dependent manner independent of HBx expression. (B) Physostigmine did not affect HBV virus production in HepG2 cells.

Supplementary Table legends

Supplementary Table 1: MTT activity after 24 hours of treatment with the indicated compounds

Supplementary Table 2: Luciferase activity after 24 hours treatment with the indicated compounds in the presence of HBx expression

Supplementary Table 3: Luciferase activity after 24 hours treatment with the indicated compounds in the absence of HBx expression