



Screening and identification of compounds with antiviral activity against hepatitis B virus using a safe compound library and novel real-time immune-absorbance PCR-based high throughput system



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ABSTRACT

There are now seven nucleoside/tide analogues, along with interferon- α , that are approved by the FDA for the management of chronic hepatitis B virus (HBV) infection, a disease affecting hundreds of millions of people worldwide. These medications, however, are limited in usefulness, and significant side effects and the emergence of viral escape mutants make the development of novel and updated therapeutics a pressing need in the treatment of HBV. With this in mind, a library containing 2000 compounds already known to be safe in both humans and mice with known mechanisms of action in mammalian cells were tested for the possibility of either antiviral activity against HBV or selective toxicity in HBV producing cell lines. A modified real-time immune-absorbance-polymerase chain reaction (IA-PCR) assay was developed for this screen, utilizing cells that produce and secrete intact HBV virions. In this procedure, viral particles are first captured by an anti-HBs antibody immobilized on a plate. The viral load is subsequently assessed by real-time PCR directly on captured particles. Using this assay, eight compounds were shown to consistently reduce the amount of secreted HBV viral particles in the culture medium under conditions that had no detectable impact on cell viability. Two compounds, proparacaine and chlorophyllide, were shown to reduce HBV levels 4- to 6-fold with an IC₅₀ of 1 and 1.5 μ M, respectively, and were selected for further study. The identification of these compounds as promising antiviral drug candidates against HBV, despite a lack of previous recognition of HBV antiviral activity, supports the validity and utility of testing known compounds for “off-pathogen target” activity against HBV, and also validates this IA-PCR assay as an important tool for the detection of anti-viral activity against enveloped viruses.

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1. Introduction

Although interferon- α and seven different nucleoside/tide analogues (NAs) have been approved for use in the management of chronic hepatitis B, these treatments are not “curative” and remain unsatisfactory (Yuen and Lai, 2011). The interferons are effective in reaching clinical endpoints in fewer than 50% (possibly even less than 20%) of those in whom they are used, and are associated with a number of undesirable side effects (Jafri and Lok, 2010; Janssen et al., 1992; Kartal et al., 2007; Lok, 1994). The NAs are HBV DNA polymerase inhibitors and are recommended for use in less than 50% of the infected population. Although, they are highly effective

at reducing viremia, the development of escape mutants and the need for long-term, possibly life-long, use limits enthusiasm. Clearly, additional anti-HBV therapeutics are needed (Stein and Loomba, 2009; Wu et al., 2012; Zoulim, 2004).

Despite the need, the cost of bringing a new drug to the market can be staggering. It has been estimated that the cost of bringing a new molecular entity from discovery to approval is on average more than \$750 million and takes at least 8 years (Weisman et al., 2006). Strategies to reduce the time between discovery of a compound's antiviral activity and its introduction to the market as a drug would be appealing, especially where commercial interest is lacking. Although there are more than 15,000 compounds that have been tested for therapeutic use in humans, many have not reached the market place (Weisman et al., 2006). There has been, however, a growing interest in “repurposing” compounds with known activity and safety profiles for new indications (Weisman et al., 2006). This strategy follows the logic that a

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molecule that has already been tested and shown to be safe in humans will require less study, time, and money to reach the market. Therefore, screening compounds with known safety profiles for uses other than their original indication could uncover candidate molecules that can more easily be developed as a therapeutic than developing a completely new drug. This strategy has already successfully been used to discover some potential therapeutics for hepatitis C virus (HCV) (Gastaminza et al., 2010). The effectiveness of this approach, of course, depends on the potency and on the illness being treated. The other advantage of this approach is that even if candidate molecules cannot be optimized and developed into therapeutics, new strategies for treating the off-target disease could be developed based on the known activity of candidate compounds.

We have developed a program to screen libraries of compounds known to be safe in people. The intent of this screening program is to identify candidates that can be used, alone or in combination, for activity against various steps in the HBV life cycle. Our initial screen, described here, measures the ability of a particular compound to reduce the amount of secreted intact viral particles in tissue culture systems known to produce and secrete infectious virus (Guo et al., 2007a; Ladner et al., 1997; Sells et al., 1988). To do this, a real-time “immune-absorbance-polymerase chain reaction” (real-time IA-PCR) assay was developed. Because this technology is specifically designed to analyze the end-point of the viral life cycle, it can identify compounds that inhibit HBV replication, nucleocapsid formation, virus assembly, and/or cellular virus transportation and secretion. This approach utilizes robotic high throughput screening in a 96-well format coupled with antibody-based capture of HBV envelope proteins and quantitative PCR to measure minimal amounts of viral DNA. By combining specific antibody-capture and qPCR we are able to confirm that we are specifically analyzing intact viral particles secreted into the culture medium of HBV producing cells and exclude subviral particles and viral intermediates.

Using this technology, a MicroSource (Gaylordsville, CT) compound library containing 2000 compounds with known safety profiles was screened. All compounds in this library are approved for animal or human use and their pharmacological and toxicological profiles have been defined and published. Furthermore, most of the compounds in this library are off-patent, so patent rights have been extinguished and the compounds can be sold without restriction. This creates the optimal situation for quickly developing an anti-HBV drug for clinical use (Kocisko et al., 2003; Weisman et al., 2006; Weissmann and Aguzzi, 2005). The strength and validity of this assay as well as the potential of the compounds identified are discussed.

2. Materials and methods

2.1. Chemicals and library screened

The chemical library spectrum from MicroSource (Gaylordsville, CT) contains 2000 compounds and is a collection intended to survey a wide range of biological activities and structural diversity for screening programs. It is further described elsewhere (Kocisko et al., 2003), and published online at <http://www.msdiscovery.com>. Each compound has a minimum of 95% purity. All compounds are dissolved in DMSO and used at a final concentration of 0.5% DMSO.

2.2. Cell culture and drug treatment

The HepG2.2.15 (G2.215) cell line, which expresses HBV from two integrated head to tail dimers of the HBV genome, was originally kindly provided by George Acs (Mt. Sinai Medical College,

New York, NY) (Sells et al., 1988). HepG2 cells were purchased from the American Type Culture Collection (Rockville, MD). The generation of the HepAD38 and HepDE19 cell line, in which HBV expression is tetracycline regulated, has been described previously (Guo et al., 2007b). HepDE19 and HepAD38 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1 mg/ml Penicillin, 100 units/ml Streptomycin (PS) and 50 µg/mL tetracycline (tet) to suppress HBV expression. HepG2 and G2.215 cells were maintained in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, Md.) supplemented with 10% FBS, with 200 µg/ml of G418 included in the medium for G2.215 cells. For drug screening, G2.215 cells were seeded in 96-well plates and grown until 60% confluent. Cultured cells were then incubated with medium containing 10 µM compounds or control medium (no drug) containing 0.5% Dimethyl sulfoxide (DMSO) the same concentration as in the medium containing drugs. After 6 days of treatment, the medium was collected for measurement of secreted enveloped HBV DNA (virion), and the cells were tested for viability as described.

2.3. Real-time Immune-absorbance-PCR to detect enveloped HBV DNA

To quantifiably detect enveloped HBV DNA (virion) in the culture medium, a method called real-time immune-absorbance-PCR (IA-PCR) was developed. Briefly, 96-well plates (Applied Biosystems, Foster City, CA) were first coated at 4 °C overnight with 250 ng per well monoclonal anti-HBs antibody (Meridian Life Science, Saco ME) diluted in 50 µl 0.01 M sodium bicarbonate coating buffer (pH 9.6). After washing with phosphate buffered saline containing 0.5% tween 20 (PBS-T), the plates were blocked with 75 µl 2% bovine serum albumin (BSA) in PBS at 37 °C for 1.5 h. Wells were again washed with PBS-T after blocking. The samples were diluted 4-fold in normal RPMI medium and 50 µl of this dilution was added to the wells and incubated overnight at 4 °C with shaking. The wells were again washed with PBS-T and all liquid was removed from the wells. PCR mastermix containing forward primer, reverse primer, FAM/TAMRA probe and 2X Universal Fast Real-Time PCR Mastermix (as per manufacturer's instructions, Applied Biosystems) was added to each well and real-time PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Two standard curves were included on each plate to assure consistency between assays.

The forward and reverse primers, with sequences 5'-ACTCGTGGTGGACTTCTCTCA-3' and 5'-AAGATGAGGCATAGCAGCAGG 3' respectively, amplify a fragment encoding a portion of the HBV small surface protein from genomic position 252nt to 432nt. The probe, located between the two primers, was labeled with the reporter dye FAM at the 5' terminus and the quencher dye TAMRA at the 3' terminus (5' FAM-TGGATGTGTCTGCGCGCTTTTATC-TAMRA3'). The reaction was performed with a 10 min incubation at 95° followed by 40 cycles of 95° for 15 s and 52° for 30 s. The C_T (Threshold cycle) value for each sample was calculated and compared with controls and standards. The reported C_T value of each compound represents the average of duplicate wells, and in some instances the C_T value is presented as HBV copy number using the standard curve of HBV DNA. Compounds causing >4-fold reduction in HBV DNA signal compared to untreated samples were considered positive anti-HBV candidates and selected for further testing.

2.4. Particle gel examination of enveloped HBV DNA

The secreted HBV virions in the culture medium were assayed as previously described (Guo et al., 2007a). Briefly, virions were precipitated from 1 ml of clarified culture medium by adding PEG8000 to a final concentration of 10% followed by incubation

at 4 °C for 1 h. The precipitates were collected by centrifugation at 10,000 rpm for 10 min and dissolved in 40 µl TNE buffer (10 mM Tris–HCl, pH7.4; 150 mM NaCl and 1 mM EDTA). The virions were directly fractionated by electrophoresis through non-denaturing 1% agarose gels and transferred to a nitrocellulose filter by blotting in TNE buffer. The DNA-containing particles on the filter were then denatured, neutralized, and the HBV DNA was detected by hybridization with a α -³²P-UTP (800 Ci/mmol, Perkin Elmer) labeled minus strand specific full-length HBV riboprobe.

2.5. Southern blot analysis of HBV replication

Intracellular HBV core DNA was extracted as described previously (Guo et al., 2007a). Briefly, cells from one well of a 6-well plate were lysed with 0.5 ml of lysis buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% NP40 and 2% sucrose at 37 °C for 10 min. Cell debris and nuclei were removed by centrifugation and the supernatant was mixed with 130 µl of 35% polyethylene glycol (PEG) 8000 containing 1.5 M NaCl. After 2 h incubation at 4 °C, viral nucleocapsids were pelleted by centrifugation at 10,000 rpm for 5 min at 4 °C, followed by 1 h digestion at 37 °C in 400 µl of digestion buffer containing 0.5 mg/ml pronase (Calbiochem), 0.5% SDS, 150 mM NaCl, 25 mM Tris–HCl (pH 8.0) and 10 mM EDTA. The digestion mixture was extracted with phenol and DNA was precipitated with ethanol and dissolved in TE (10 mM Tris, pH8.0, 1 mM EDTA) buffer. Half of the DNA sample was resolved by electrophoresis into a 1.5% agarose gel. The gel was then soaked in 0.2 N HCl for 10 min and then subjected to denaturation in a solution containing 0.5 M NaOH and 1.5 M NaCl for 1 h, followed by 1 h neutralization in a buffer containing 1 M Tris–HCl (pH 7.4) and 1.5 M NaCl. DNA was then blotted onto Hybond-XL membrane (GE Healthcare) in 20X SSC buffer. HBV DNA was examined by hybridization with α -³²P-UTP labeled probe specific for HBV.

3. Results

3.1. Specificity and sensitivity of real time immune-absorbance-PCR

The goal of this work was to screen compound libraries for activity against hepatitis B virus (HBV), however, infectivity assays for HBV in tissue culture are technically difficult and not very robust. Because of this an efficient method to measure decreases in the amount of enveloped particles containing viral DNA secreted from HBV producing cells, such as G2.215 cells, was developed. It is reasoned that since production and release of enveloped viral DNA, or virions, by G2.215 cells requires that all synthetic steps in the viral life cycle be functional except entry, drugs suppressing any post-entry stage of the HBV life cycle can be identified by measuring levels of secreted virion in culture medium. By utilizing an antibody to first capture virions based on their envelope protein and then specifically amplifying HBV DNA, we are excluding analysis of subviral particles and viral intermediates and ensuring specific analysis of infectious virions. To take advantage of this, a high-throughput screening system was developed, allowing us to robotically administer our compound library in 96-well microtiter plates. The sensitivity of real time PCR combined with the specificity of immune-absorbance allows for detecting the small amounts of secreted virion and differences in virion levels that would be expected from the low number of cells within each well of a 96-well plate.

The linearity, reproducibility and dynamic range of this assay were first evaluated to determine the strength and validity of the assay (Fig. 1). Successful PCR, resulting from the amplification of DNA associated with HBs captured by the immune-absorbance

step, was shown to be specific for HBV virions and did not result in non-specific amplification (Fig. 1A). Because a lower C_T value in a real time PCR assay indicates that the level of DNA amplification reached the set threshold value earlier in the reaction, a lower C_T value can be interpreted as a greater amount of starting HBV DNA present in the sample (Materials and Methods). With this in mind, the results show the lowest C_T value, 27.47, was amplified in medium from untreated G2.215 cells (Fig. 1A, no drug). Replacing the anti-HBs antibody in the immune-absorbance portion of the assay with an irrelevant anti-His tag antibody resulted in a loss of PCR signal, with a C_T value of 36 meaning it was below the threshold of detection as determined by the standard curve (Fig. 1A, anti-his AB). The specificity of detection was further supported by the fact that no HBV DNA was amplified from medium for HepG2 cells (C_T of 36), which are the parental cells of the G2.215 cells and do not produce HBV (Fig. 1A, HepG2). Suppression of HBV replication by treatment of cells with the known HBV inhibitor lamivudine (3TC) resulted in a dose-dependent decrease of HBV DNA signal in the assay (Fig. 1A, 3TC treated). Incubation of G2.215 cells with varying concentrations of 3TC for 6 days, the time point optimized for drug incubation in this assay, resulted in a dose dependent suppression of the captured HBV DNA with a maximum reduction in C_T value of ~ 3.3 (Fig. 1A), or ~ 8 -fold when converted to viral DNA copies (Fig. 1B). Together these results demonstrate the specificity of the assay to successfully pull down and amplify HBV DNA without isolating or amplifying non-specific DNA products.

In addition to the specificity, this assay is also sensitive enough to detect as few as 30 copies of HBV genome (Fig. 1C). Using a standard curve of HBV DNA, the linear range of the assay was determined to be from 30 copies to 3×10^6 copies of HBV genome (Fig. 1C). Thus, at the standard conditions used, the C_T differences are large enough to distinguish between viral levels in the medium of the cells producing HBV from that in the “negative” non-producing control (G2.215 versus HepG2 cells). Similar results were obtained using the HepAD38 cell line, which produces intact HBV virions under the control of a tetracycline-responsive promoter (Ladner et al., 1997; Zhu et al., 2009). HepAD38 cells produce HBV virus derived from transcripts from an integrated transgene after removal of tetracycline. HBV DNA is amplified with a C_T of 35.1, which is near the lower limit of detection, when the HBV transgene is repressed, while transgene activated cells have a C_T of 26.7 (Suppl. 1). For ease of use, and because consistent results were seen in both systems, G2.215 cells were used as the positive control in these studies.

3.2. The Z' value for robotic high-throughput screening

To determine the extent to which the assay lends itself to the high-throughput screening platform, the Z' value of the assay was determined, as defined previously (Muller et al., 2008; Zhang et al., 1999). In this assay, the Z' value reflects the well-to-well variations in assay results with regard to the presence or absence of HBV DNA. To determine the Z' value of our assay, 48 wells of a 96 well plate were utilized to measure HBV DNA levels from G2.215 cell culture medium and the other half of the plate (48 wells) was used to measure HBV DNA from HepG2 cell culture medium (Fig. 2). The average C_T values and standard deviation (SD) of positive wells (with HBV) and negative wells (without HBV) were determined and the Z' value was calculated. The Z' value of this assay is calculated as 0.801 (Fig. 2), and since Z' values of greater than 0.7 are generally considered adequate for robotic high-throughput screens, the real-time IA-PCR assay described here meets this threshold and was advanced for further use (Muller et al., 2008; Zhang et al., 1999).

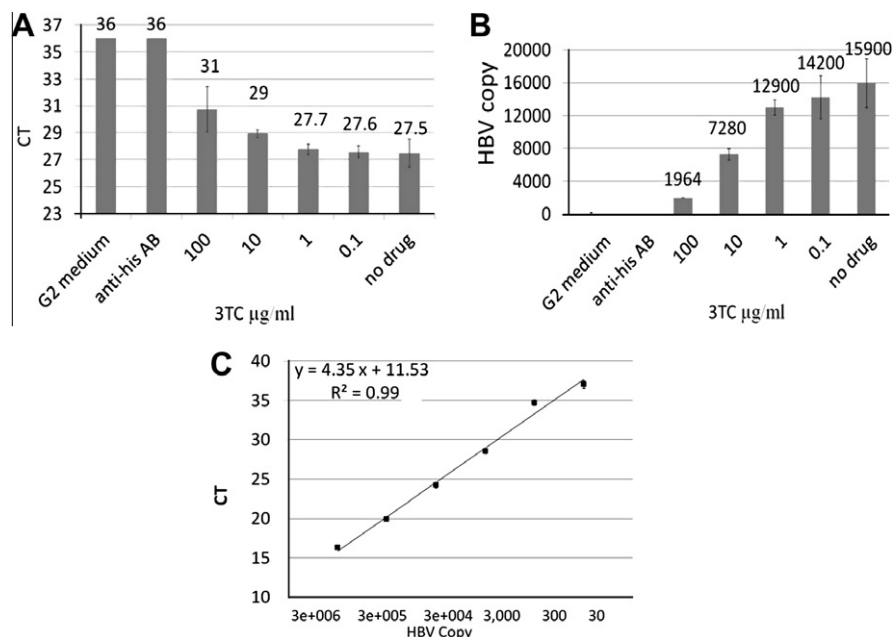


Fig. 1. Real-time IA-PCR specifically detects enveloped HBV DNA in G2.215 culture medium. G2.215 cells were seeded in 96-well plates and incubated with lamivudine at different doses for 6 days. Cell culture medium was incubated with anti-HBs pre-immobilized on 96-well PCR plates at 4 °C overnight. HBV DNA in captured virions was then amplified by real-time PCR. Medium from HepG2 cells and G2.215 cell medium incubated in wells coated with an anti-His antibody instead of anti-HBs were used as negative controls. (A) A dose-dependent increase in C_T value was seen in medium from cells incubated with lamivudine. (B) The effect on C_T levels was converted to reduction of HBV DNA by correlating C_T to virus copy number using a standard curve of HBV genomic DNA. (C) Serial dilution of HBV genomic DNA served as the standard curve for the real-time PCR assay.

Determining Z' value for IA-PCR assay	
$Z' = 1 - \frac{([3 \times \text{SD of "+" samples}] + [3 \times \text{SD of "-" samples}])}{(\text{AVE "+" samples} - \text{AVE "-" samples})}$	
$0.801 = 1 - \frac{([3 \times 1.88] + [3 \times 0])}{(28.31 - 0)}$	
If $1 > Z' > 0.9$	Considered an excellent assay
If $0.9 > Z' > 0.7$	Considered a good assay
If $0.7 > Z' > 0.5$	Hit selection will be significantly from any improvement
If $Z' = 0.5$	Minimum recommend for high throughput screening

Fig. 2. The Z' value of the real-time IA-PCR as a high-throughput screening platform was determined. The equation for calculation of Z' value is shown. The Z' value of this assay was determined by the average (AVE) and standard deviation (SD) of positive controls (+) and negative controls (–). Averages represent data from half of the wells (48) of a 96 well plate for both positive and negative samples. Medium from G2.215 cells was used as the positive control, and medium from HepG2 cells, which do not produce HBV, was used as the negative control.

3.3. Screening results overview

After verification, the assay was used to screen a commercial compound library with ~2000 compounds with known safety profiles and mechanisms of action (Microsource Inc. described in Materials and Methods) for drugs that lowered the amount of HBV DNA detected in the culture medium. Briefly, G2.215 cells were seeded in 96-well plates and incubated with either “carrier” (the 0.5% DMSO medium in which the compounds were dissolved), 30 μM lamivudine (as a positive control for inhibition of HBV) or 10 μM of each of the test compounds from the library. After 6 days of treatment, cell culture medium was collected for use in the real-time PCR assay as described in the Materials and Methods, and

illustrated in Fig. 3A. Duplicate wells were run for each sample and the average C_T value corresponding to each compound was calculated. Two standard curves, one with a 1:4 serial dilution of medium from G2.215 cells treated with carrier alone (Fig. 3C) and another with a 1:10 serial dilution of HBV genome copies from plasmid P13 (Lamontagne et al., 2010), were included on each IA-PCR plate for calculation of level of reduction of HBV signal and copy number of HBV DNA respectively. While it would be ideal to run each sample in triplicate, the cost of supplies and reagents made this prohibitive.

As expected from the assay development stages (Fig. 1), undiluted medium from cells treated with carrier alone amplified to a C_T of ~26, and this signal was reduced, proportionally, with 1:4 serial dilutions (Fig. 3C), indicating the linearity and dynamic range of the assay. Also as expected, levels of HBV DNA in medium from HepG2 cells were below the level of detection, confirming the specificity seen during assay design and setup (Fig. 3C and Fig. 1). In addition, medium from G2.215 cells incubated with lamivudine had a C_T of ~30–31 (Fig. 3B, Sample 43), indicating a significant reduction in HBV DNA compared to cells treated with carrier alone. This is important because this not only confirms the ability of the assay to detect compounds which successfully reduce HBV DNA levels, but also defines the level of reduction that could be expected from candidate drugs during the screen of this compound library. A C_T of 30, representing a 4-fold reduction in HBV DNA, was therefore used as a cut-off to define primary hits in the assay. The viability of cells was determined by MTT assay of cell culture plates used for drug treatment after media collection (data not shown).

Fig. 3B shows the results of a typical IA-PCR plate, where 3–4 of the 40 compounds run per plate usually met our criteria of a “hit”. Compounds with high variation (SD) were not counted as hits regardless of their average C_T value. An example of such a sample would be Sample 36 in Fig. 3B. The names of the screened compounds are listed in Suppl.2.

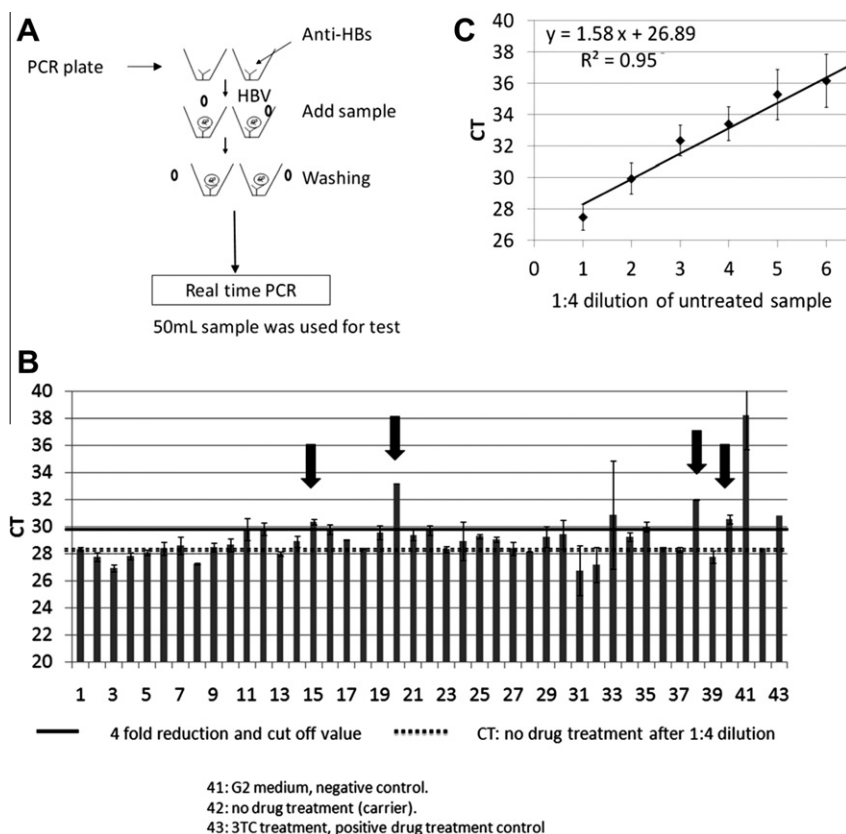


Fig. 3. IA-PCR assay setup and typical results. (A) Work-flow describing the procedure for the real-time IA-PCR assay. (B) Representative data shows the typical results from an assay plate. Samples were diluted 1:4 from culture medium before being added to the plate in duplicate. After immune-absorbance, enveloped HBV DNA was amplified by real-time PCR. The solid black line across the data represents a 4-fold reduction compared to the untreated control. The dotted line represents the C_T value of the untreated control, diluted 1:4 along with the treated samples. Arrows indicate compounds that reduced HBV DNA >4-fold, and are examples of samples that were selected for retesting. (C) The standard curve for the complete drug screening assay, derived cumulatively from the 1:4 serial dilutions of untreated, positive control medium run on the 24 independent assays. This was used as a reference for reduction of secreted HBV DNA in each assay.

From the screen of 2000 compounds, 153 were scored as hits in the primary screen. Each primary hit that was found by MTT test to be non-cytotoxic was retested in a second run. During retesting eight compounds were confirmed as effective in reducing HBV DNA and their names as well as reported functions and mechanisms are shown in the Table 1. One of these identified hits, cycloheximide, is a known inhibitor of protein synthesis and inducer of

cell stress (Paoletti et al., 2010). Because of its known mechanism, the reduction of HBV DNA in cycloheximide treated cells most likely was caused by non-selective cellular toxicity and thus was not pursued further. Therefore, seven compounds were ordered for re-synthesis and their activity was confirmed in dose response assays using the same real-time IA-PCR assay (Fig. 4A and B). Proparacaine and chlorophyllide showed the most significant and

Table 1

Candidate compounds that showed a dose-dependent effect on HBV DNA in treated G2.215 cells upon retesting.

Compound name ^a	Fold HBV reduction ^b	Approved or intended use ^c	Mechanism ^d	Reference
Proparacaine	6	Eye drop, Anti-HSV	Prevent viral envelopment; reduce pain and discomfort	Romanowski et al. Storey et al.
Chlorophyllide	4	Anti-cancer including liver cancer, anti-HSV	Interaction with a carcinogen or removal of toxic aflatoxins	Breinholt et al. Egner et al.
Natamycin	4	Anti-mycotic agents	Suppress microorganism growth	Maher et al.
Raloxifene	3.5	Anticonvulsant	Estrogen receptor modulators	Rey et al.
Aminohippuric Acid	3	Renal function diagnosis	Organic ion transporter	Morisaki et al.
Pipamperone	3.8	Commercial name: dipiperon antipsychotic	Inhibitor of G protein-receptor	Van Craenenbroeck et al.
Cycloheximide	2	Protein synthesis inhibitor	Suppression of protein synthesis, cell stress	Greenberger et al.
Dimercaprol	3	Chelating agent (As, Au, Hg antidote)	Interfere protein interaction	Morris et al.

^a G2.215 cells were incubated with 10 μ M of indicated compound for 6 days. No cytotoxicity was observed at this concentration.

^b The amount of HBV DNA in the culture medium was determined by IA-PCR, using HBV specific primers, immune-absorbance of an aliquot of medium with mAb to HBV envelope protein. Methods are described in text.

^c Approved or intended use is the use for which the compound has received US FDA approval (a) or for which it has been used in animal or human trials.

^d Mechanism is the action attributed to the compound as stated on either its "label" or literature.

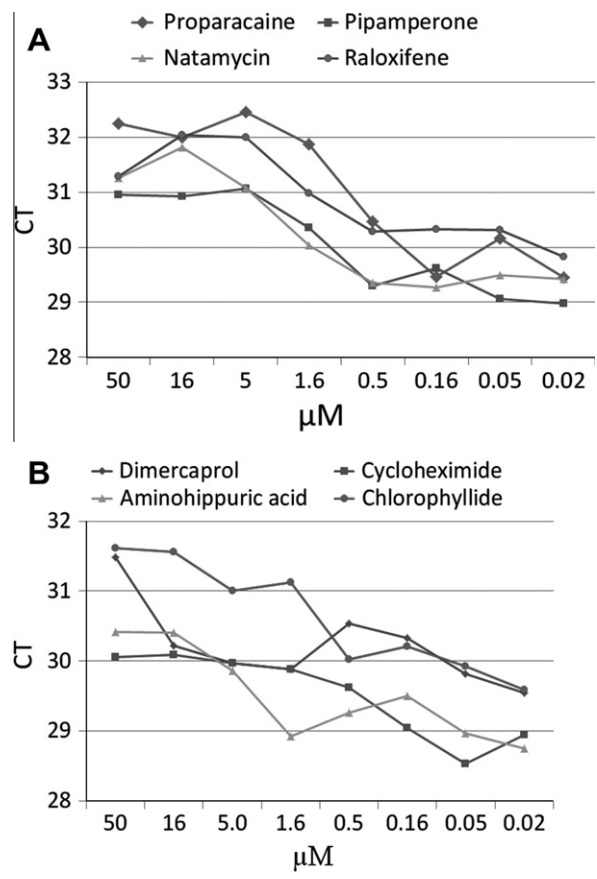


Fig. 4. Eight candidate compounds were confirmed by retesting using a dosage gradient. G2.215 cells were incubated with a serial dilution of candidate compound and the C_T value was determined using the IA-PCR assay. (A) IA-PCR results for the first sample set including the candidate drug, proparacaine, one of two compounds selected for further testing. (B) IA-PCR results for the second sample set, including chlorophyllide, the second compound selected for further testing.

consistent activity in this system. At a concentration of 5 μ M, proparacaine reduced HBV virion levels in culture medium by $\sim 3 C_T$ ($P < 0.01$), whereas chlorophyllide reduced the amount of HBV enveloped virus by $\sim 2 C_T$ at the same concentration ($P < 0.05$) (Fig. 4A and B). The IC_{50} for proparacaine against HBV was 1.0 μ M, and was 1.5 μ M for chlorophyllide (Fig. 4A and B). Therefore, these two candidates were evaluated further.

3.4. Secondary assays to confirm antiviral activity of compounds identified for the ability to reduce enveloped HBV DNA in a cell culture system

Further study suggested that proparacaine reduced enveloped virus in culture medium after 6 days of treatment, but did not reduce intra-cellular HBV DNA levels. Unlike empty spherical particles, intact HBV virions contain the large surface protein (LHBs). Therefore, the presence and amount of LHBs was examined by western blot using the anti-LHBs monoclonal antibody MA18/7 (Lu et al., 1996). The reduction of LHBs usually suggests the reduction of viral particles, though filamentous particles also have LHBs. Fig. 5A shows that 6 day treatment with 10 or 20 μ M proparacaine inhibited secretion of HBV particles containing LHBs in culture medium ~ 3 - to 4-fold, which agrees with the results seen in the primary screens.

To determine whether proparacaine inhibits HBV replication, cellular HBV DNA was isolated from G2.215 cells after treatment as described previously (Lu et al., 1996). HBV replication intermediates were analyzed by Southern blot with a labeled HBV probe. Compared to the untreated control, no reduction in the level of relaxed circular HBV DNA (RC-DNA, Fig. 5B) was seen at any concentration tested. There was some evidence of a reduction in double stranded linear (DSL) and single stranded DNA in the 10 μ M sample, although this was not consistently seen in other experiments (Fig. 5B). These potential reductions were modest, and more work is needed to determine if this is indeed a definitive effect. These results, however, support the idea that proparacaine may work on post-replication stages perhaps by preventing HBV envelopment and/or secretion.

To analyze the impact of chlorophyllide on HBV we used non-denaturing particle gel analysis. Because G2.215 cells do not produce enough HBV virions for this assay, we used HepDE19 cells. HepDE19 is a stable cell line expressing HBV under the control of tetracycline, allowing the secretion of high levels of HBV viral particles after removal of tetracycline. Similar to G2.2.15 cells, HepDE19 cells also secrete naked nucleocapsids into the medium for unknown reasons (Guo et al., 2011, 2007b). Under non-denaturing conditions the naked nucleocapsids can be separated from the virions based on size. HBV DNA both in virions and nucleocapsids can then be examined by Southern blot using a labeled HBV-specific probe. After a 5 day treatment with chlorophyllide, HBV virions and nucleocapsids were resolved on a 1% non-denaturing agarose gel, transferred to a nylon membrane and hybridized with a labeled HBV-specific probe (Guo et al., 2007b). Fig. 6A suggests that

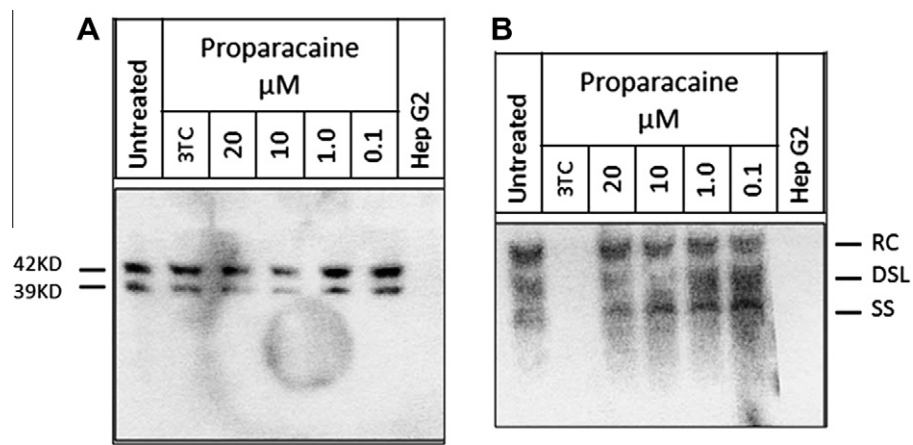


Fig. 5. Western and Southern blot analysis of the role of proparacaine in reducing HBV DNA. G2.215 cells were treated with different doses of proparacaine. (A) Western blot detecting LHBs. (B) Southern blot detecting cellular HBV replication intermediates. RC, relaxed circular; DSL, double-stranded linear; SS, single-stranded DNA.

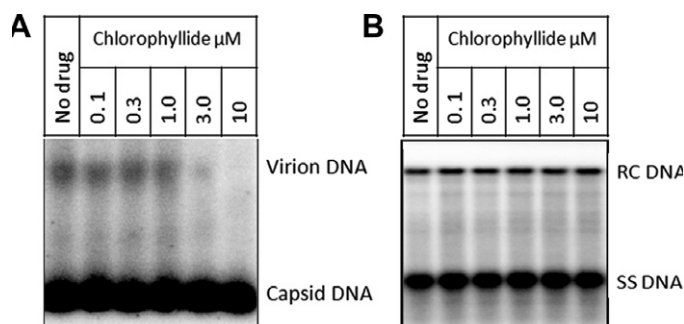


Fig. 6. Particle gel and Southern blot analysis of the role of chlorophyllide in reducing HBV DNA. HepDE19 cells were cultured with Tet- medium for 5 days to initiate HBV expression. Cells were then treated with chlorophyllide at different doses. (A) HBV virions and nucleocapsids in culture medium were resolved in a 1% non-denaturing gel and transferred to membrane. The membrane was then hybridized with a HBV-specific probe. (B) Southern blot to detect cellular HBV DNA.

treatment with chlorophyllide reduces viral particles in the cell culture medium in a dose-dependent manner, however, the formation and secretion of nucleocapsids were not affected. Analysis of cellular HBV DNA by Southern blot suggested that chlorophyllide did not inhibit HBV replication. Treatment with up to 10 μM chlorophyllide did not repress either RC or SS DNA levels (Fig. 6B). The anti-viral activity of chlorophyllide and its analogies have been extensively studied and published elsewhere (Guo et al., 2011). These results support the idea that, like proparacaine, chlorophyllide may also prevent HBV virion formation and/or secretion.

4. Discussion

There are a growing number of examples where libraries of compounds known to be safe in humans are being tested for the possibility of activity for indications other than those for which they were intended. This phenomenon of “repurposing” has, in addition to the library described here, been applied to other screens of safe compound libraries (Bader and Korba, 2010; Gastaminza et al., 2010; O'Connor and Roth, 2005; Thomas et al., 2009). This approach is particularly appealing in the field of HBV where current therapies are lacking, and it was with this in mind that the current screen was performed.

The high throughput screening system, utilizing the real time IA-PCR assay described, is both highly sensitive and highly specific. Because of the PCR platform for this assay, the sensitivity is high enough to detect as few as 30 copies of HBV DNA. In addition, the 96-well plate format allows large numbers of compounds to be quickly screened and lends itself to robotic handling. Moreover, this assay has the ability to identify not only drugs which inhibit HBV replication such as the nucleoside analog lamivudine, but also can identify drugs such as proparacaine and chlorophyllide which likely work on post-replication stages such as viral assembly, transport and/or secretion. The principle used in this technology, first capturing protein associated virus by use of an immobilized antibody, followed by amplification and detection of viral DNA, can easily be applied to screen for inhibitors of other enveloped viruses or pathogens.

Two compounds proparacaine and chlorophyllide were identified to have activity against HBV in this screen. Although their mechanisms still require additional study, the data suggests they may work on post-replication stages of the HBV life cycle. The proparacaine family members all have a common propylbenzoate core structure (Fig. 7A). Chlorophyllide is a chlorophyll derived from a compound named chlorin, the core structure of which is a large heterocyclic aromatic ring consisting of three pyrroles and one pyr-

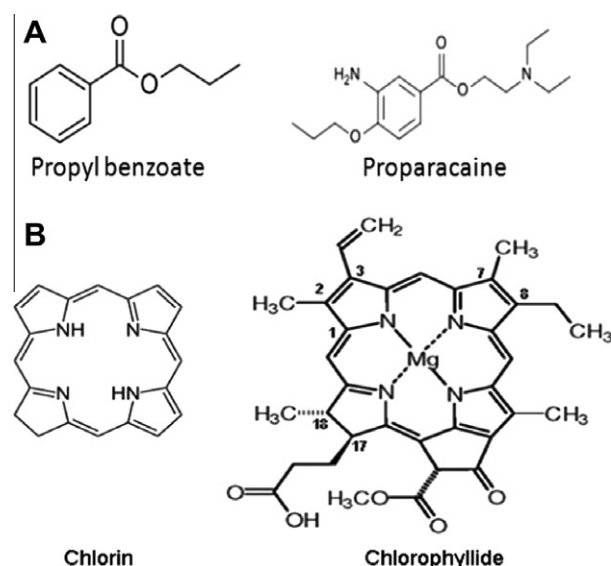


Fig. 7. The structure of selected candidate compounds. (A) The structure of proparacaine, along with the core structure for the family of compounds is presented. (B) The structure of chlorophyllide, along with the core structure for its analogs is presented.

roline coupled through four methine linkages (Fig. 7B). Magnesium-containing chlorins are called chlorophylls (Godnev et al., 1963). Both of these compounds as well as their analogs have previously been used in humans or animals. Proparacaine, known commercially as Alcaine, is an approved drug used primarily as an ophthalmic anesthetic (as eye drops) to reduce pain and discomfort. Chlorophyllide is an alkylated porphyrin, structurally related to chlorophyll. The most studied chlorophyll is chlorophyllin, which has anticancer activity that has been shown to be effective against liver cancer (Reinbothe et al., 2006; Sarkar et al., 1994). Although the mechanism is still unclear, the interaction of chlorophyllin with a carcinogen or the removal of toxic aflatoxins may play an important role in its anticancer activity (Breinholt et al., 1995; Egner et al., 2001; Guo et al., 2011).

The antiviral activity of these two compounds has previously been reported. For example, chlorophyllide has been reported to inactivate Varicella Zoster Virus and influenza virus (Mekler et al., 1969), and proparacaine can inactivate equine herpesvirus-4 and feline herpesvirus-1 (Romanowski et al., 1999; Storey et al., 2002). Neither of these compounds, however, can inactivate nonenveloped viruses such as feline calicivirus (Storey et al., 2002), suggesting that the antiviral activity of these two compounds is associated with viral envelopment. This is consistent with our observations that the antiviral mechanism of these two compounds is dependent on preventing the envelopment or secretion of the virus. In vitro, the infectivity of HSV and influenza virus is lost following treatment with chlorophyllide and proparacaine, which implies that these two drugs might also prevent viral entry (Mekler et al., 1969; Romanowski et al., 1999; Storey et al., 2002). Based on these findings, further research is currently underway to investigate the effect of these two compounds on HBV infectivity using an in vitro infection system.

5. Conclusion

We have successfully established a novel high throughput screening technology, real-time IA-PCR, based on the capture of viral particles by an antibody immobilized on a PCR plate and then subsequent assessment of viral load by real-time PCR. Using this technology to screen 2000 compounds we were able to identify

eight compounds that were shown to consistently reduce the amount of secreted HBV viral particles in the culture medium under conditions that had no detectable impact on cell viability. The anti-HBV activity of two of these, proparacaine and chlorophyllide, which were shown to reduce HBV levels 4- to 6-fold with an IC_{50} of 1 and 1.5 μ M respectively, have been further confirmed by other technologies. Additionally, the basis for this assay makes it easily adapted to the identification of compounds against other enveloped viruses by simply using different antibodies specific for the envelope of the virus being studied.

Disclosure

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.antiviral.2013.02.001>.

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Glossary

HBV: Hepatitis B virus
HCV: Hepatitis C virus
IA-PCR: immune-absorbance PCR
LHBs: HBV large surface protein
NAs: nucleotide analogues
PCR: polymerase chain reaction