P450s under Restriction (PURE) Screen Using HepaRG and Primary Human Hepatocytes for Discovery of Novel HBV Antivirals

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ABSTRACT: Herein is reported a novel screening paradigm PURE (P450s under restriction) for the identification and optimization of hits as part of a hepatitis B virus (HBV) antiviral discovery program. To closely represent *in vivo* hepatocytes, differentiated HepaRG cells (dHRGs) and primary human hepatocytes (PHHs) were used as the basis for an HBV infection system. However, a significant challenge arose during potency evaluation in using cultured dHRGs and PHHs as screening platforms because, as with hepatocytes *in vivo*, these cells express active cytochrome P450 enzymes and thus can metabolize test compounds. The observed antiviral effects may be the cumulative result of a dynamic pool of parent compound and metabolites thus confounding structure activity relationship (SAR) interpretation and subsequent optimization design initiatives. We show here that PURE methodology restricts metabolism of HBV-infected dHRGs and PHHs and thus provides highly informative potency data for decision-making on key representative antiviral compounds.

KEYWORDS: P450, Hepatitis B virus, Metabolism, Screening, Antiviral compounds, Capsid assembly modulators

Hepatitis B virus (HBV) infection is a major cause of chronic liver disease that affects more than 240 million people and effective therapy continues to be a global unmet medical need. The persistence of HBV infection is enabled by the stable episomal covalently closed circular DNA (cccDNA) which is converted from the partially double stranded relaxed circular DNA (rcDNA) that is encapsidated within enveloped virions.¹

In general, to address the complexity of disease across therapeutic areas, cell-based assays^{2–5} have proven to be a vital tool in the discovery of new drugs and an impressive array of cell lines have been designed to optimize disease-focused drug discovery. For hepatitis B, *in vitro* models established in primary human hepatocytes (PHHs)⁶ and differentiated HepaRG cells (dHRGs)⁷ are highly useful as platforms for HBV drug discovery since they are permissive to HBV infection and reproduce a viral life cycle that is dependent on cccDNA. These better represent *in vivo* hepatocytes compared to hepatoma cell lines as both dHRGs and PHHs express hepatic markers and retain intact immune response pathways.^{8–10} In addition, dHRGs and PHHs have been utilized to evaluate the quantitative pharmacokinetic,^{11,12} metabolic,^{13,14} and toxicologic profiles^{15–18} of advanced candidate compounds during discovery and optimization stages. The resulting data enable dosing regimen, biomarker, and safety requirements to be established. In addition to passive permeability barriers and transporter pumps, both dHRGs

and PHHs express an extensive functional array of oxidative, reductive, and conjugative enzymes that may metabolize test compounds.

Inhibition of P450s in dHRGs has been well-characterized by both isozyme selective and nonselective agents and reduction of the latent cytochrome P450 activity in dHRGs using P450 inhibitors has been reported to quantitate and modify pharmacokinetic end points as well as to decipher hepatic toxicologic outcomes.^{19,20} Conversely, P450 metabolic stimulation has been reported wherein dHRGs maintained CYP3A4 and CYP1A2 metabolic capability and responded to inducers.²¹ The induction/inhibition of a wide variety of CYPs, conjugative enzymes, and efflux transporters in dHRGs and PHHs has also been reported.^{22,23} In analogous work using PHHs, the nonspecific, irreversible P450 inhibitor 1-amino-benzotriazole (ABT) was used to modulate metabolism as an approach to decipher the mechanism of cytotoxic activity of the known human hepatotoxic aflatoxin B1.²⁴

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Figure 1. CYP3A4 expression and P450 metabolic activities in HRG cells. (A) pHRGs, dHRGs, and PHHs were immunostained with anti-CYP3A4 antibody (red), and nuclei were stained with DAPI (blue). (B) Relative metabolic activities of CYP isoforms from dHRG. (C) Metabolic activities of CYP isoforms in pHRGs, dHRGs, and PHHs (batch 4038 and 4119E). RLU, relative light units, levels of luciferin normalized to no cell control. Statistical significance between pHRG and dHRG/PHHs was evaluated using the two-tailed unpaired *t* test (***, $p < 0.001$; ****, $p < 0.0001$).

Although preserving metabolic activity during test compound evaluation may provide some desirable aspects, it was decided to test whether inhibition of P450 activity in dHRGs would provide insight and decisive impact on compound prioritization decisions for potential HBV antiviral agents. It was expected that many test compounds, particularly leads discovered early during the project and hence not optimized for physical properties or metabolic stability, would be vulnerable to metabolism. These metabolically susceptible compounds could be converted to primary, secondary, and even reactive metabolites to generate an undesired, dynamically changing assay mixture composed of parent and metabolites whose potency would be a complex, time-dependent summation of all active entities thus confounding interpretation of the data.

The goals of this work were 2-fold: first, to establish a straightforward and reproducible P450 inhibition protocol that could be readily applied for both routine antiviral assays and high-throughput screening using HBV-infected dHRGs without affecting HBV biomarkers or enhancing nonspecific cytotoxicity; second, to evaluate whether limiting the P450-mediated metabolism of test compounds would provide a direct evaluation of the potency of the parent compound

unconfounded by metabolism. Herein, our efforts are presented on the development of a novel **PURE** (P450s under restriction) assay protocol²⁵ including the results on key HBV capsid assembly modulators (CAMs) using both standard and **PURE** conditions in HBV-infected dHRGs.

HepaRG cells have gained importance as an *in vitro* cell-based system for antiviral research due to key limitations with the availability and use of PHHs.^{8,10,26,27} Upon differentiation, HRGs resemble PHHs by providing membrane permeability barriers and efflux transporters and are crucially able to metabolize test compounds via P450 enzymes.^{28–33} To establish the **PURE** condition, the baseline metabolic activities of dHRGs were monitored. Expression of CYP3A4 in dHRGs was examined by using an anti-CYP3A4 antibody and compared with PHHs (Figure 1A). Consistent with previous observations,³⁴ CYP3A4 was detected in both dHRGs and PHHs but absent from the nondifferentiated proliferating HRGs (pHRGs). The metabolic activities of the major P450 enzymes in dHRGs were further assessed by using the nonlytic P450 assays where cell-permeable luminogenic substrates are converted to a luciferin product upon metabolism such that the level of luciferin is proportional to the metabolic activity of the CYP enzymes.³⁵ Among the P450 isoforms, CYP3A4 showed

the highest relative metabolic signal (>100-fold over background), followed by CYP1A2 and CYP2C9 (>10-fold over background signals) while relative activities for CYP2B6 and CYP1A1/1B1 were lower (<10-fold over background signals, Figure 1B). The metabolic activities of CYP1A2, 2C9, and 3A4 from dHRGs were also compared with those determined in PHHs. Results showed that dHRGs and PHHs displayed similar relative metabolic activities among the three CYP isoforms (Figure 1C). In contrast, the nondifferentiated pHRGs were much less metabolically active, displaying a >10-fold lower CYP1A2, >100-fold lower CYP2C9, and >200-fold lower CYP3A4 metabolic activity compared to dHRGs and PHHs.

With the CYP metabolic activities of dHRGs confirmed, it was of interest to verify if metabolism remained stable and dependent on HBV infectivity status. The metabolic activities were monitored over time because establishment of cccDNA-dependent HBV replication would require more than 1 week to reach a plateau after HBV infection.^{7,36} Results showed that the metabolic activities of CYP1A2, 2C9, and 3A4 in dHRGs and PHHs were stable for at least 19 days under the current cultured conditions (Figure 2A,B). To determine if HBV infection would affect metabolism, the relative activities of CYP isoforms from uninfected and infected dHRGs were measured on day 11 postinfection at which time persistent viral infection had been established. In the study, it was shown that HBV infection did not significantly alter the metabolic activities of CYP1A2, 2C9, and 3A4 (Figure 2C).

The next study aim was to establish a method to restrict metabolism in HBV-infected dHRGs or PHHs during treatment with antiviral compounds while maintaining the biological integrity of the inhibitory potency screen. Three key criteria for a useful metabolically restricted system were defined: (1) Metabolism should remain restricted over the course of antiviral compound treatment; (2) Restriction of metabolism should not impact HBV replication; (3) Cell health should not be compromised.

During screening and profiling, antiviral compounds were added to the infected cells starting on day 5 postinfection, replenished on day 8, and their activities were measured on day 11 postinfection. To attain the PURE system criteria that metabolism should remain restricted over the course of antiviral treatment, the ability of known, nonselective P450 inhibitors 1-aminobenzotriazole (ABT) and SKF-525A (proadifen) to reduce CYP activities in HBV-infected dHRGs was examined using the scheme in Figure 3A. dHRGs were infected with HBV on day 0, after which increasing concentrations of ABT or SKF-525A were incubated with infected dHRGs for 1 h on day 5 and day 8 postinfection. After the 1 h incubation, media containing P450 inhibitors was removed, cells were replenished with fresh media, and the incubation was continued. The effect of ABT and SKF-525A treatment on CYP3A4, 2C9, and 1A2 activity was determined on day 11 postinfection. Both ABT and SKF-525A inhibited the activities of CYP1A2, 2C9, and 3A4 in a dose-dependent manner. ABT was more effective at inhibiting CYP1A2 and CYP3A4 compared to CYP2C9 (Figure 3B) whereas SKF-525A was most efficient at inhibiting CYP3A4 compared to the other two CYP isoforms (Figure 3C). Furthermore, treatment of cells with ABT and SKF-525A for 1 h on day 5 and day 8 postinfection did not induce cytotoxicity (Figure 3B,C).

The next study was directed at determining whether combining ABT and SKF-525A could further increase CYP

Figure 2. dHRGs and PHHs remained metabolically active over 19 days in culture. CYP activities were measured in (A) dHRGs and (B) PHHs postdifferentiation or cell-seeding, respectively. (C) Metabolic activities from dHRGs with or without HBV infection determined on day 11 postinfection. RLU, relative light units, levels of luciferin normalized to no cell control. Statistical significance between the metabolic activities in the uninfected (no HBV) and infected (+HBV) groups was evaluated using the two-tailed unpaired *t* test (ns, not significant).

inhibition. Compared to single P450 inhibitor treatment using the corresponding concentration, treatment of dHRGs with 10 mM ABT in combination with 50 μ M SKF-525A was very effective and inhibited the three CYP isozymes by 82–90%. Furthermore, retreatment of cells with ABT and SKF-525A on day 8 postinfection was necessary to maintain CYP inhibition as the single treatment on day 5 alone did not durably inhibit all three CYPs. Table 1 summarizes the results of ABT and SKF-525A, alone and in combination, and the time of addition effect against the metabolic activities of CYP3A4, 1A2, and 2C9 in dHRG cells.

The next challenge for PURE addressed whether CYP restriction would adversely affect HBV viral replication and cell viability. In addition to dHRGs, PHHs infected with HBV were also treated with ABT and SKF-525A. Consistent with the results from dHRGs, the activities of CYP1A2, 2C9, and

Figure 3. Restriction of metabolic activity using ABT and SKF-525A. (A) HBV infection and CYP inhibitor treatment scheme. Infected dHRG cells were treated with increasing concentrations of (B) ABT or (C) SKF-525A for 1 h on day 5 and day 8 postinfection. Cytotoxicity (cytotox) and CYP activities were measured on day 11 postinfection. Statistical significance was compared between the samples treated with the highest concentrations vs the lowest concentrations using the two-tailed unpaired *t* test (**, *p* < 0.01; ***, *p* < 0.001; ****, *p* < 0.0001; ns, not significant).

Table 1. Inhibition of CYP P450 Enzymes in dHRGs Treated with 10 mM ABT and 50 μM SKF-525A, Alone or in Combination, for 1 h on Day 5 Only or on Both Day 5 and Day 8

CYP	ABT		SKF-525A		ABT + SKF-525A	
	CYP inhibition ^a (%)		CYP inhibition ^a (%)		CYP inhibition ^a (%)	
	day 5 only	day 5 and 8	day 5 only	day 5 and 8	day 5 only	day 5 and 8
1A2	37.1 ± 14.1	91.0 ± 3.1	2.2 ± 10.0	30.8 ± 14.9	39.2 ± 2.6	88.8 ± 5.0
2C9	−9.4 ± 4.1	67.5 ± 12.4	21.4 ± 8.1	42.2 ± 7.3	36.9 ± 15.1	82.1 ± 14.7
3A4	9.6 ± 12.2	87.8 ± 8.0	80.3 ± 11.3	93.0 ± 1.9	83.3 ± 11.5	90.3 ± 7.5

^aData represent mean values and standard deviations from at least 3 data sets.

Figure 4. Effect of ABT and SKF-525A (SKF) on CYP activities, HBsAg, and cell viability (CV) using infected dHRGs and PHHs. ABT and SKF were added to infected (A) dHRGs or (B) PHHs on days 5 and 8 postinfection for 1 h. CYP activities, HBsAg, and CV were measured on day 11 postinfection.

3A4 were inhibited by ABT and SKF-525A in HBV-infected PHHs (Figure 4A,B). The secreted HBV surface antigen (HBsAg) was monitored as a biomarker for HBV replication, and cell viability was measured by monitoring intracellular ATP levels. Results showed that neither HBsAg nor cell viability in infected dHRGs was adversely affected by treatment with the ABT and SKF-525A combination (Figure 4A). HBV-

infected PHHs appeared to be slightly more sensitive to CYP inhibitor treatment with levels of HBsAg and cell viability reduced by about 20% and 10%, respectively (Figure 4B). No significant reduction in HBsAg and cell viability was observed with infected PHHs when the SKF-525A concentration was reduced to 25 μM (Figure 4B). Thus, dHRG and PHH cell replication and viability were maintained following treatment

Figure 5. Antiviral activity of HBV CAMs in dHRGs with or without CYP restriction. (A) HBV infection and drug treatment scheme. (B–E) Cells were treated with or without 50 μ M SKF-525A and 10 mM ABT prior to the addition of test compounds. HBV DNA inhibition dose response curves and structures of CAMs: (B) Bay 41-4109, (C) GLS4, (D) NVR 3-1983, and (E) AT-130 were determined in the presence (dotted lines, ■) or absence (solid lines, ●) of CYP inhibitors. Statistical significance of the HBV DNA inhibition data (EC_{50} values) between the CYP restricted (■) and non-CYP restricted (●) groups was determined by using the two-tailed unpaired t test (*, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant).

Table 2. Antiviral Activity of HBV CAMs against HBV DNA Replication and Their Stability in dHRGs and PHHs, with or without P450 Restriction (CypR)

compound ^a	HBV DNA EC_{50} [μ M]		HBV DNA EC_{50} fold change	$T_{1/2}$ [min]	
	no CypR	CypR		no CypR	CypR
Bay 41-4109	0.22 $\pm$ 0.081	0.26 $\pm$ 0.15	0.85	189	431
GLS4	0.59 $\pm$ 0.31	0.011 $\pm$ 0.0071	54	7.7	>560
NVR 3-1983	2.1 $\pm$ 0.43	0.20 $\pm$ 0.10	11	20.5	159
AT-130	5.7 $\pm$ 1.5	0.78 $\pm$ 0.20	7.3	2.9	>560

^aHBV CAMs did not inhibit cell viability up to the highest concentration tested ($CC_{50} > 30 \mu$ M). EC_{50} values against HBV DNA represent mean and standard deviations from at least 3 data sets.

for 1 h on days 5 and 8 with the combination of ABT and SKF-525A.

With PURE conditions for CYP inhibition now established in HBV-infected dHRGs and PHHs, four representative HBV capsid assembly modulators (CAMs) Bay 41-4109 (Figure 5B), GLS4 (Figure 5C), NVR 3-1983 (Figure 5D), and AT-130 (Figure 5E) were examined to evaluate the impact of P450

inhibition on antiviral potency.^{37–39} Infected dHRGs were first treated with or without the combination of 10 mM ABT and 50 μ M SKF-525A on day 5 postinfection. After the 1 h incubation with ABT and SKF-525A, the media containing CYP inhibitors was washed out and replaced with media containing different concentrations of HBV CAMs and the incubation with the infected cells continued for 3 days. The 1 h

Figure 6. Hits identified from a phenotypic screen using HBV-infected dHRGs under **PURE** or metabolically active conditions. (A) **PURE** condition: infected dHRGs were treated with ABT and SKF-525A prior to the addition of test compounds (20 μ M). (B) Metabolically active condition: infected dHRGs were treated with test compounds (20 μ M). HBsAg and cell viability were determined. Hit compounds (green) were defined as those with >50% HBsAg inhibition and <20% cell viability inhibition.

treatment with CYP inhibitors, followed by the 3 day incubation with the test compounds, was repeated on day 8 postinfection (Figure 5A). At the end of the incubation period, i.e., day 11 postinfection, the levels of HBV DNA inhibition were measured.

The resulting data showed that when CYP metabolism was restricted under **PURE**, the potencies of NVR 3-1983, AT-130, and GLS4 were enhanced by 11-fold, 7.3-fold, and 54-fold, respectively, compared to testing without metabolic restriction (Table 2). In contrast, Bay 41-4109 showed similar EC_{50} values against HBV DNA under both **PURE** and metabolically active conditions.

To confirm that the observed potency values under **PURE** conditions were related to metabolic restriction, the stability of Bay 41-4109, NVR 3-1983, AT-130, and GLS4 was determined in PHHs in the presence and absence of the combination of ABT and SKF-525A. As shown in Table 2, Bay 41-4109 was the most stable of the four compounds in hepatocytes and exhibited only a 2.3-fold increase in $t_{1/2}$ under **PURE** conditions. Accordingly, the EC_{50} values under standard and **PURE** conditions for Bay 41-4109 were very similar at 0.22 and 0.26 μ M, respectively. On the other hand, GLS4, NVR 3-1983, and AT-130 showed significant improvement in antiviral activities under **PURE** conditions consistent with the increased metabolic stability.

For GLS4, NVR 3-1983, and AT-130 under standard assay conditions, the “apparent” antiviral activity of the parent was diminished by conversion of each parent to less active/inactive metabolites. Our conclusion was that suppression of CYP metabolism could reveal the potency of the parent CAM and that the enhanced potency values under **PURE** demonstrated that metabolism of the parent was impacting the antiviral activity. A one-to-one correspondence of stability to antiviral potency change would not be expected across a large set of compounds since both active and inactive parents could afford both active and inactive metabolites.

As demonstrated, **PURE** provided an insightful distinction of inherent potency differences among representative HBV CAMs based on modulation of their metabolic stability. This alternative potency order, along with the insight into metabolic effects from conducting both the standard and the **PURE**

assay, demonstrates that metabolic stability was a key factor for these compounds and thus offers highly relevant information for prioritization decisions on optimization.

To investigate whether **PURE** evaluation of a large and diverse screening collection would provide enhanced insight into project discovery decisions, a set of about 10 000 compounds selected from an in-house, druglike library was screened at a concentration of 20 μ M in HBV-infected dHRGs using either standard or **PURE** conditions. To detect active compounds that could potentially suppress cccDNA functions, HBsAg was used as a surrogate marker of HBV cccDNA inhibition, and compounds with >50% HBsAg inhibition and <20% cellular toxicity were considered hits in the screen. In both screens, a majority of the test compounds showed <50% HBsAg reduction, cytotoxic activity, or an increase in the levels of HBsAg to provide results that did not meet the hit criteria and compounds were not further pursued (Figure 6). A total of 171 compounds, representing a 1.7% hit rate, were identified as active hits from the screen using **PURE** (Figure 6A). Interestingly, most of these active compounds, about 85% of them, were not identified as hits when the same set of compounds was screened using metabolically active HBV-infected dHRGs (Figure 6B). These contrasting results illustrate that many parent compounds that were unstable in the presence of metabolically active CYP isoforms were not identified as actives due to metabolic conversion to an inactive/less active compound(s).

A similar noteworthy finding was that, among the hits identified using the **PURE** screening condition, 8.8% of those compounds had been incorrectly eliminated as hits based on cytotoxicity in the screen using metabolically active dHRGs. Thus, over the 10 000-compound test set, **PURE** offered a distinctive selection of hits based on both potency and cytotoxicity measures compared to metabolically corrupted data from a screen employing dHRGs that retained CYP activities.

It could be argued that hepatocyte enzyme-mediated metabolic transformation of a parent test compound is desirable during screening as a “real-world” check on how it will perform *in vivo*. Three points would argue against that expectation. First, metabolic conversion of the test compound

will generate a dynamic mixture of parent and metabolites whose composition will be time-dependent, and therefore, readouts of potency and cytotoxicity will be a complex summation of parent and all active molecules that were generated. The potency and cytotoxicity readouts will be dependent on many factors including test compound and metabolite concentrations as well as P450 and transporter profiles, and this dynamic metabolic mixture will be in constant flux until metabolism, or the assay, is ended. It seems unlikely that this dynamic event could be easily and exactly reproduced. If the parent compound is appreciably metabolized, it will be difficult and time-consuming to prove what components of this unstable metabolic mixture were responsible for the observed potency and efficacy. Second, metabolic conversion may be poorly mimicked in isolated cells where metabolites are continually trapped, and clearance is limited allowing successive rounds of metabolism. Third, medicinal chemistry design and structure activity relationship (SAR) exploration will operate more efficiently when potency and cytotoxicity data are provided on the parent test compound exclusively and not on a dynamic metabolic mixture.

The PURE methodology described has provided a rapid, reproducible, and decisive approach to quantitate the potency and cytotoxicity of parent compounds using either a large screening set or a more refined group of optimized candidates. CYP inhibition has been demonstrated to be a fundamental feature of compound evaluation and that should not come as a surprise. One can anticipate that a wider foray into the focused inhibition of oxidative, conjugative, and transport processes occurring in cells during compound screening and optimization would be highly beneficial to ensure appropriate data for compound prioritization decisions. Such control of those cellular metabolic processes demonstrated by PURE would further the essential goal of providing potency and cellular toxicity evaluation uniquely for the parent compound. Finally, PURE and standard assay formats may be employed together to provide an enriched data set with complementary information to assess lead compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00630>.

Experimental details, reagent descriptions, and assay protocols (PDF)

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■ Author Contributions

G.D.H. and S.D.K. contributed the concept of PURE, design of NVR 3-1983, and selection of the HTS library. A.M.L. contributed to the design and analysis of the antiviral studies, high-throughput screening, and the CYP restriction studies using HRGs and PHHs. C.E. contributed to designing, performing, analyzing the CYP expression/inhibition studies, and evaluating HBV CAMs with and without CYP inhibitors. All authors contributed to writing the manuscript and approved the final version.

■ Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PURE, P450s under restriction; (HBV), hepatitis B virus; dHRGs, differentiated HepaRG cells; PHHs, primary human hepatocytes; SAR, structure activity relationships; cccDNA, covalently closed circular DNA; rcDNA, relaxed circular DNA; ABT, 1-aminobenzotriazole; SKF-525A, proadifen; CAMs, capsid assembly modulators; pHRGs, proliferating HRGs; CYP P450, cytochrome P450; HBsAg, secreted HBV surface antigen; ATP, adenosine triphosphate; $t_{1/2}$, the time required to reduce compound concentration by 50%; EC₅₀, the concentration of compound that induces a response halfway between baseline and maximum

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