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Hepatoselective Dihydroquinolizinone Bis-acids for HBsAg mRNA Degradation

Nicky Hwang,[#] Liren Sun,[#] Daisy Noe, Patrick Y. S. Lam, Tianlun Zhou,* Timothy M. Block,* and Yanming Du*

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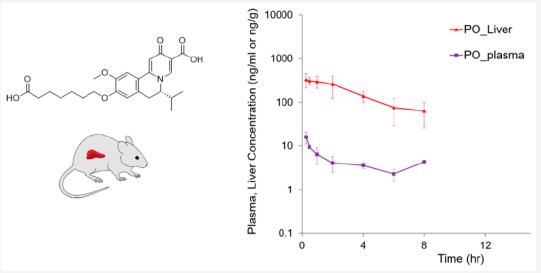
ABSTRACT: Chronic hepatitis B (CHB) is characterized by high levels of hepatitis B virus (HBV) surface antigen (HBsAg) in blood circulation. A major goal of CHB interventions is reducing or eliminating this antigenemia; however, there are currently no approved methods that can do this. A novel family of compounds with a dihydroquinolizinone (DHQ) scaffold has been shown to reduce circulating levels of HBsAg in animals, representing a first for a small molecule. Reductions of HBsAg were a result of the compound's effect on HBsAg mRNA levels. However, commercial development by Roche of a DHQ lead compound, RG-7834, was stopped due to undisclosed toxicity issues. Herein we report our effort to convert the systemic RG7834 compound to a hepatoselective DHQ analog to limit its distribution to the bloodstream and thus to other body tissues.



KEYWORDS: *Chronic hepatitis B (CHB), hepatitis B virus (HBV) surface antigen (HBsAg), dihydroquinolizinone (DHQ), hepatoselective distribution, PAPD 5 and 7, organic anion transporting poly peptide protein 1B1 (OATP1B1), OATP1B3*

A high level of hepatitis B virus (HBV) surface antigen (HBsAg) in the serum of patients is a common feature of chronic hepatitis B (CHB),[1] which infects 258 million people worldwide and causes ∼880 000 deaths annually due to cirrhosis, hepatocellular carcinoma (HCC), and liver failure.[2] The reduction of HBsAg antigenemia (HBsAg in the blood) has become one of the three goals for the primary end point of CHB therapy, along with the reduction of viremia and the normalization of blood-level liver-derived transaminases.[3,4] This is in part because HBsAg, in addition to being a protein essential for completion of the viral life cycle, is also believed to play a role in immunosuppression and the maintenance of the chronic infected state.[5,6] However, although the current standard of care medications with either pegylated interferon alpha or nucleos(t)ide analogues (NUCs) can suppress viral replication, none reliably induce the loss of HBsAg.[7] There is thus a significant need to develop new HBV therapeutics.

There are a number of investigational HBV therapeutics in the development pipeline.[8] Small interfering RNA (siRNA) is promising, but its human use requires multiple parenteral injections, and even then, reductions in HBsAg are usually not more than 1 to 2 logs, possibly because of the limited penetration of infected hepatocytes.[8] Nucleic acid polymers (NAPs) have shown promise in reducing circulating HBsAg and eliciting HBsAg antibodies, but the efficacy is limited, coadministration with interferons and NUCs is necessary, it requires multiple injections, and its mechanism of action is uncertain.[9,10]

Recently, a dihydroquinolizinone compound, RG-7834, has been reported to reliably reduce the levels of multiple HBV gene products, including HBsAg and HBeAg, as well as HBV DNA (Figure 1), in tissue cultures and in animal models. For example, oral treatment with RG-7834 in HBV-infected uPA-SCID mice harboring human hepatocytes resulted in a 1 log reduction of HBsAg in the circulation.[11] Moreover, in woodchucks chronically infected with woodchuck hepatitis virus (WHV), oral administration with RG-7834 induced multilog reductions of both WHV and surface protein in the blood.[12] The mechanism of action of RG7834 is now known. It is an inhibitor of the cellular (viral host) Polyadenylating Polymerases 5 and 7 (PAPD 5 and 7).[13−17] PAPD5 and 7 are noncanonical polyadenylating polymerases that mediate short adenylations and provide a signal for the degradation of aberrant cell transcripts and the maturation of a subset of noncoding transcripts.[13−17] We and others have found that PAPD5 and 7 inhibition is the basis of its anti-HBV activity. The fact that the inhibition of PAPD5 and 7 with DHQ causes a reduction of HBV RNA levels is surprising and suggests that HBV mRNA behaves very differently from most host mRNA.

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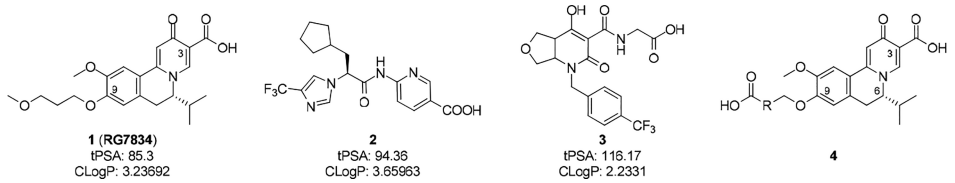
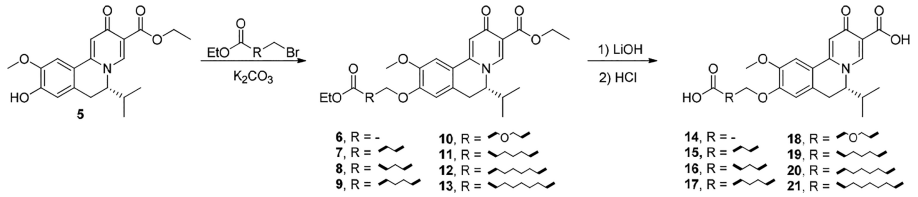


Figure 1. Structures of RG7834, two known hepatoselective molecules, and our proposed DHQ derivative **4** with an additional acid group through position 9.

Scheme 1. Synthesis of Bis-acids 14−21



This provides a novel opportunity for antiviral drug development.

Attracted to this novel chemotype and unique mechanism, several companies and institutions have generated patent applications based on the RG7834 structure, published in the last 5 years since the initial reports of RG7834.[18−26] However, the development of RG7834 has been met with difficulties because of toxicity concerns, especially the acute neurotoxicity liabilities observed from RG7834.[26] Recent patent applications claim structural variations around the substituents and the fused tricyclic RG7834 frame with chemistry-accessible approaches, but the majority of them still pursued systemic DHQ derivatives and had little information on safety issues,[18−25] except for neurotoxicity concerns.[26]

Because PAPD 5 and 7 are cell enzymes involved in the synthesis and decay of host RNA,[15] an effect upon host functions from the systemic use of a PAPD5/7 inhibitor is not surprising. Thus, having drugs that are more selective for liver hepatocytes, which are the cells targeted by HBV, is one way to minimize or eliminate unnecessary side effects resulting from the inappropriate distribution of RG7834 to other tissues. Therefore, hepatoselective DHQ compounds should have great potential to improve the safety of this novel family of anti-HBV compounds.

However, RG7834 was generated in the pursuit of traditional drug-like properties (systemic use with better absorption, distribution, and bioavailability) for oral anti-HBV treatment.[11] RG7834 showed very good permeability with a Papp(A − B) of 12.8 × 10^{-6} cm s^{-1} and an efflux ratio of 1.3 in the Caco-2 assay. The mouse single-dose pharmacokinetics (PK) profile of RG7834 demonstrated that RG7834 has moderate plasma clearance (Cl = 41.9 mL min^{-1} kg^{-1}), good distribution, satisfactory oral exposure (oral bioavailability, F = 62%), and marginal liver/plasma distribution. Considering the toxicity to neurite formation,[26] a good distribution to the plasma and other organs may actually be unnecessary and undesired. Therefore, contrary to the regular medicinal practice of pursuing a highly systemically bioavailable lead compound, we focused on developing DHQ compounds that have low to moderate bioavailabilities but high liver exposure and liver/plasma ratios.

Several distinct medicinal chemistry approaches have emerged in other disease fields to exploit the specific receptors or binding sites on the surface of liver cells, which can then facilitate the liver-targeted delivery of small molecules. Examples include transporter-mediated active uptake,[27,28] the prodrug strategy,[29,30] and the address-and-message approach.[31] Because of the abundant expression of Organic Anion Transporting Polypeptide protein 1B1 (OATP1B1) and 1B3 (OATP1B3) on liver hepatocytes, these proteins provide opportunities to explore liver-targeted drug delivery.[32] This strategy has successfully produced clinical candidates such as glucokinase activator **2** for the treatment of type 2 diabetes (Figure 1)[33] and hypoxia-inducible factor prolyl hydroxylase (HIF-PHD) inhibitor **3** (Figure 1).[34]

The presence of an acid group at a correct position is believed to be a common recognition element for OATP transporters.[27] RG7834 already has an acid group at position 3, but its good permeability and oral bioavailability indicate that RG7834 is a systemic molecule rather than a liver-targeting molecule.[11] We envisioned increasing the polar surface area (PSA) of RG7834 and modulating the LogD or cLogP (as these compounds are dissociable acids) of the RG7834 derivatives to change their absorption, distribution, metabolism, and excretion (ADME) to increase their liver tropism and result in less plasma exposure (low F). PSA is an important physicochemical property that is shown to correlate well with human intestinal absorption, Caco-2 monolayer permeability, and blood−brain barrier (BBB) penetration.[35,36] PSA is often described as topological polar surface area (tPSA), which can be easily calculated. The tPSA of RG7834 is 85 A^2, which is <90 A^2, suggesting the potential to penetrate through the BBB.[37] Thus, new DHQ derivatives with tPSA of >90 A^2 will be pursued to avoid the unnecessary BBB penetration. Previous structure−activity relationship (SAR) studies have revealed that position 9 of RG7834 can tolerate a variety of groups with slight to moderate activity changes,[11] but the introduction of an ADME-modifying group at this position has not been extensively investigated, and although a few instances of an acid group at this position through a linker are present in several patents,[18,22] it has not been reported specifically as a recognition element of OATP transporters. Herein we report the discovery of a novel series of hepatoselective DHQ compounds with bis-acid moieties (**4**, Figure 1) with liver-specific transport proteins OATP1B1 and OATP1B3 substrate properties.

To synthesize the DHQ derivatives for SAR studies, we started with an intermediate **5** that was used in the preparation

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A

Cmpd	tPSA	cLogP/LogD	EC_{50} (μM)	CC_{50} (μM)
14	113.4	2.2	> 10	> 10
15	113.4	2.9	1.01 +/- 0.35	> 10
16	113.4	3.1	0.38 +/- 0.10	> 10
17	113.4	3.7	0.087 +/- 0.022	> 10
18	122.6	2.9	2.78 +/- 0.71	> 10
19	113.4	4.2/-0.65	0.023 +/- 0.011	> 10
20	113.4	4.7/0.07	0.0076 +/- 0.00083	> 10
21	113.4	5.2/0.70	0.0046 +/- 0.00074	> 10
RG7834	85.3	3.2/1.39	0.0018 +/- 0.00016	> 10

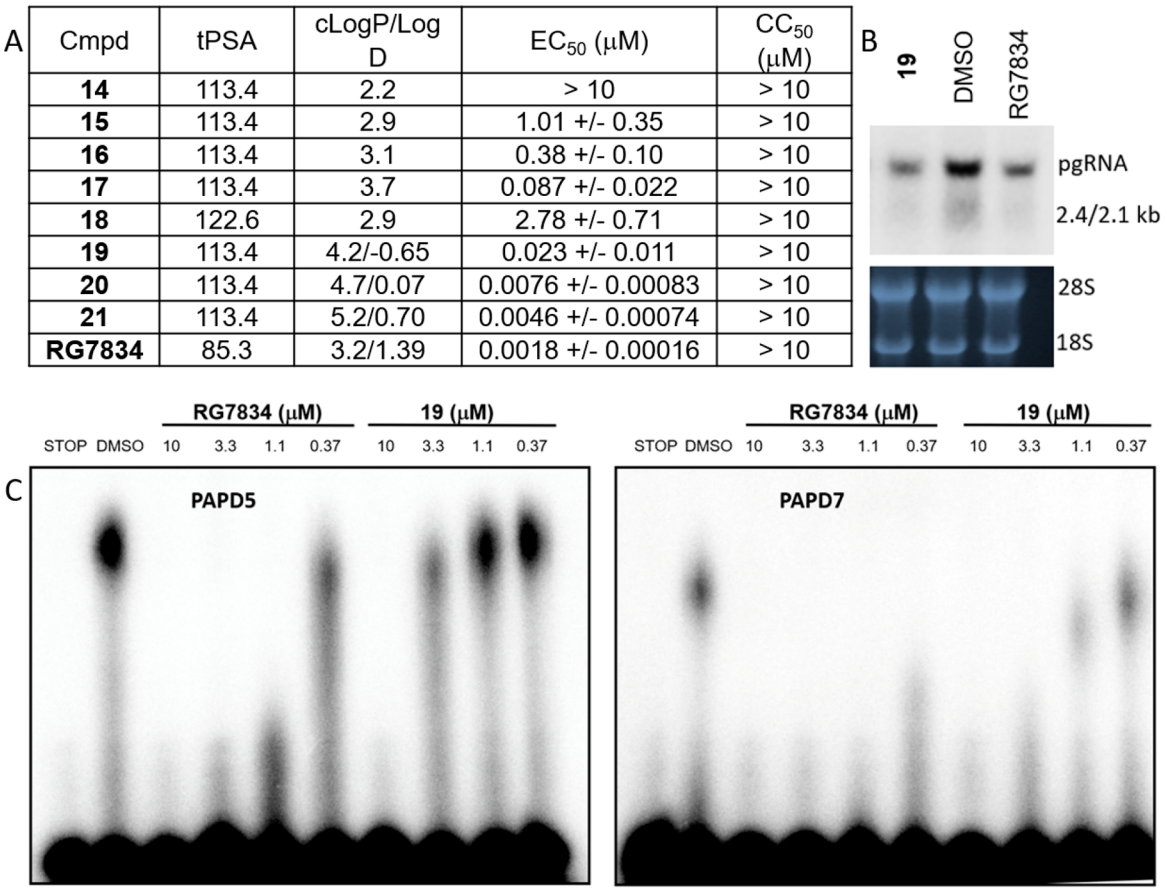

Figure 2. (A) Activities of new DHQ derivatives to reduce HBsAg (EC_{50}). (B) Northern blot analysis of bis-acid **19** for HBV mRNA reduction (at 1 μM after 5 days of treatment). (C) Dose-dependent inhibition of **19** against PAPD **5** and 7 compared to RG7834.

A

Cmpd	OATP1B1		OATP1B3	
	Uptake Ratio (-/+ inhibitor)	Uptake Ratio ($Transporter_{(-inhibitor)}$/$Mock_{(-inhibitor)}$)	Uptake Ratio (-/+ inhibitor)	Uptake Ratio ($Transporter_{(-inhibitor)}$/$Mock_{(-inhibitor)}$)
19	11.17	18.99	11.57	24.80
RG7834	0.38	0.71	0.42	0.56

B

Cmpd	P_{app} (A-B) 10^{-6} cm/s	P_{app} (B-A) 10^{-6} cm/s	Efflux Ratio	BBB Penetration Potential
19	0.38	0.55	1.47	Low P_{app} (A-B) < 3
RG7834	9.17	35.77	3.90	Moderate P_{app} (A-B) ≥ 3, and 10 > efflux ≥ 3

Figure 3. (A) Substrate determination of **19** on OATP1B1 and OATP1B3. Estradiol 17-β glucuronide was used as a positive control. (B) BBB penetration potential in MDCK-MDR1 cells.

of RG7834 derivatives.[11,18−20] O-Alkylation provided bis-esters **6**−**13** with new side chains of different lengths containing additional carboxylic esters. Upon hydrolysis, the resulting bis-acids **14**−**21** were generated (Scheme 1).

These compounds were first evaluated in HepG2.2.15 cells. HepG2.2.15 is a human liver hepatoblastoma cell line. The HBV dimer was artificially integrated into the cell genome so that it could stably produce all HBV gene products. It is routinely used to evaluate anti-HBV drugs. RG7834 is an inhibitor of PAPD 5 and 7, which is necessary for the stable accumulation of HBV mRNA. We therefore compared the ability of the new DHQ derivatives to reduce the amount HBV mRNA and the associated HBsAg in HepG2.2.15 cells. The results from a 4-day treatment showed that the linker composition and the length of the linkers (C1−C8) between the DHQ core and the introduced acid appear to significantly influence the DHQ derivatives' ability to inhibit HBsAg levels. Bis-acid **14**, with the shortest linker, does not show activity for the reduction of HBsAg, but as the linker increases in size, the activities improve accordingly (**15**−**17**, **19**−**21**, Figure 2A).

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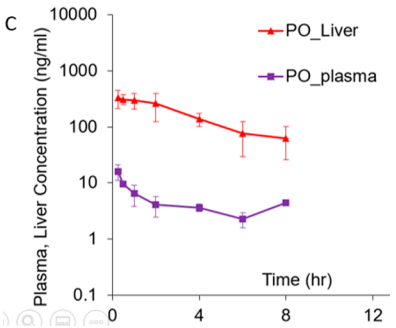
A

Liver Microsomal Stability				Caco-2			CYPs
Human		Mouse					
Cl (μL/min/mg)	$T_{1/2}$ (min)	Cl (μL/min/mg)	$T_{1/2}$ (min)	P_{app} (A-B) (10^{-6}, cm/s)	P_{app} (B-A) (10^{-6}, cm/s)	Efflux Ratio	IC_{50} against 9 isozymes
0	∞	0.8	1736	0.39	2.06	5.30	> 10 μM

B

Route	Dose (mg/kg)	Cl (mL/min/kg)	V_{ss} (L/kg)	$T_{1/2}$ (hr)		C_{max} (ng/mL)		AUC_{last} (h*ng/mL)		F (%) In plasma
				Plasm	Liver	Plasm	Liver	Plasm	Liver	
IV	3	198	3.26	1.48				255		
PO	8				3.54	16.1	333	34.7	1307	5.1

C



D

Time (hr)	0.25	0.5	1	2	4	6	8
[C] in liver (ng/mL)	333	309	300	261	138	76	63
[C] in liver (nM)	730	680	660	570	300	170	140
C_{max} ratio (liver/plasma)	20.7						
AUC_{last} ratio (liver/plasma)	37.7						

Figure 4. (A) ADME evaluation of **19**. (B) PK profile of **19** through IV and PO routes. (C) Liver versus plasma distribution of **19** in the PO route. (D) Concentration of **19** in the liver over a 8 h time course.

The activities improve to a low nanomolar EC_{50} as the number of linker carbons reaches and passes six (**19**–**21**, Figure 2A). Interestingly, compound **14** and compound **21** have the same tPSA (113.4), but the activity differs by more than 2000-fold. Moreover, to avoid a potential fatty acid β-oxidation, the carbon atom at the position β to the carboxylic acid in **17** was replaced with an oxygen atom. The resulting **18** increased the tPSA and reduced the cLogP, but this change led to a three-fold loss of activity, suggesting that the cellular activity was not driven by tPSA but by other parameters like cLogP or LogD. An early lead compound **19** was found to have an EC_{50} of 23 nM in this assay, reduce HBV mRNA (pregenomic RNA, subgenomic 2.4 kb RNA, and 2.1 kb RNA) in a Northern blot analysis like RG7834 (Figure 2B), and inhibit PAPD5 and PAPD7 in a dose-dependent manner like RG7834, indicating that the bis-acid **19** has the same anti-HBV mechanism as RG7834 (Figure 2C).[16]

Compound **19** (DHQ-E-OH) was selected to showcase the pharmacological changes from our structural modifications to RG7834. First, whether the new bis-acids can be absorbed into hepatocytes was evaluated in HEK293 cells transfected with either an OATP1B1 or OATP1B3 transporter and in vector control HEK293 cells in the presence or absence of an inhibitor, estradiol 17-β glucuronide. **19** was judged by the uptake ratio in each pair of cell lines. A compound is defined as a potential substrate of the corresponding transporter in these assays when both ratios are bigger than 2. **19** was found to have all of the ratios in both cell lines higher than 10, indicating that it is a substrate for both OATP1B1 and OATP1B3 (Figure 3A). In contrast, RG7834 was unsurprisingly determined to not be a substrate for either transporter because the uptake ratios for RG7834 were all < 2, indicating that the presence of an acid group at position 3 in RG7834 is not at a correct position for it to be a substrate of an OATP (Figure 3A). Comparing the structure of **19** to that of RG7834, it is obvious that the additional pendant acid moiety from position 9 provided the new molecule with the properties of being an OATP1B1 and OATP1B3 substrate. In addition, to address the central nervous system (CNS) safety concerns, **19** was evaluated in MDCK-MDR1 cell monolayers as a surrogate model[38,39] for BBB penetration potentials together with RG7834. The results clearly demonstrate that **19** has a lower probability of crossing the BBB and thus causing neurotoxicity safety concerns (Figure 3B). **19** possesses a low risk, whereas RG7834 has a moderate risk with a high penetration rate (P_{app}(A − B) of 9.17 × 10^{-6} cm s^{-1}, Figure 3B).

ADME evaluations revealed that **19** is very stable in both human and liver microsomes, with half lives longer than 28.9 h. It is not an inhibitor against nine Cyp enzymes tested (IC_{50} > 10 μM), and as desired, its permeability (Papp(A − B)) in the Caco-2 assay was reduced to 0.39 × 10^{-6} cm s^{-1}, from 12.8 × 10^{-6} cm s^{-1} for RG7834, suggesting poor permeability (Figure 4A). To confirm the in vitro observations of OATP-mediated uptake in an in vivo setting, **19** was evaluated in male CD1 mice following intravenous (iv) and oral (po) administration. **19** displayed low plasma exposure after the oral route (bioavailability F = 5.1%, Figure 4B) in mice. However, as shown in a parallel liver PK study with the same oral dose, **19** showed much higher exposure in the liver than in plasma (assessed by the area under the liver/plasma concentration–time curve (AUC), Figure 4C,D), with an average ratio of 37.8 over 8 h. When compared at different time points (Figure 4D), **19** was quickly absorbed into the liver and steadily reduced in the liver, indicating that a hepatoselective distribution was achieved through the installation of an additional acid group to RG7834, most likely via hepatic uptake mediated by OATP isoforms.

Carboxylic acid is a common pharmacophore shown in many U.S. Food and Drug Administration (FDA)-approved drugs. There are more than 450 marketed drugs containing the carboxylic acid functional group.[40] By contrast, compounds containing bis-acid functional groups are rarely seen, especially as systemic drugs. However, several drugs with bis-acid functional groups are in clinical use, such as enalaprilat,

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methotrexate, and nexletol. The ionization characteristics of bis-acids may not allow for sufficient GI absorption, but this can be improved through a prodrug approach or chemical modification to balance the physicochemical properties. Overall, GI absorption, hepatoselective distribution, and toxicity improvement should be considered in a balanced manner for the molecular design against liver diseases.

In summary, on the basis of the structure of RG7834 and the analysis of its ADME and PK profiles, we have incorporated an additional acid group into the side chain of RG7834 at position 9. Through the increase in tPSA and the modulation of the cLogP/LogD of the new molecules, we have identified compound **19** to be potent in both the PAPD 5 and 7 enzyme assays and HBV mRNA degradation cellular assay. Further evaluation showed that unlike RG7834, **19** is a substrate of both OATP1B1 and OATP1B3, which may facilitate the absorption of **19** into the liver. This in vitro result was translated into an in vivo setting: **19** demonstrates much better hepatoselective distribution in a mouse PK study than RG7834, with an average liver/plasma ratio of 37.8 over 8 h. More importantly, bis-acid **19** demonstrated a low risk for crossing the BBB in comparison to the moderate risk of RG7834.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00228.

Synthesis procedure of intermediate **5** and analytical data (^{1}H NMR, MS) for the intermediates and product, including supercritical fluid chromatography (SFC) for chirality determination; synthesis of RG-7834 from intermediate **5**; comparison of activity for commercially purchased RG-7834, in-house version, and the reported value from Roche; synthesis procedure and analytical data (^{1}H NMR, MS) for the DHQ derivatives reported in this Letter, compounds **14**−**21**; description of the procedures for testing PAD5/7 enzymatic inhibition; description of the procedures for cellular assays; description of the procedures for the distribution coefficient and the kinetic solubility measurement; description of the procedures and the data for testing the compounds to see if they are substrates of OATP1B1 and OATP1B3 transporters; description of the procedures and the data for testing the BBB penetration potentials; description of the procedures and the data for the metabolic stability study; description of the procedures and the data for testing Cyp inhibition; description of the procedures and the data for the Caco-2 permeability study; and description of the procedures for the single-dose PK/toxicity study (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Tianlun Zhou − *Baruch S. Blumberg Institute, Doylestown, Pennsylvania 18902, United States*; Email: tianlun.zhou@bblumberg.org

Timothy M. Block − *Pennsylvania Biotechnology Center of Bucks County, Doylestown, Pennsylvania 18902, United States; Hepatitis B Foundation, Baruch S. Blumberg Institute, Doylestown, Pennsylvania 18902, United States*; Email: tim.block@bblumberg.org

Yanming Du − *Medicinal Chemisty, Baruch S. Blumberg Institute, Doylestown, Pennsylvania 18902, United States*; orcid.org/0000-0002-4665-8348; Email: yanming.du@bblumberg.org

Authors

Nicky Hwang − *Baruch S. Blumberg Institute, Doylestown, Pennsylvania 18902, United States*

Liren Sun − *Baruch S. Blumberg Institute, Doylestown, Pennsylvania 18902, United States*

Daisy Noe − *Baruch S. Blumberg Institute, Doylestown, Pennsylvania 18902, United States*

Patrick Y. S. Lam − *Lam Drug Discovery Consulting, LLC, Chadds Ford, Pennsylvania 19317, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsmedchemlett.1c00228

Author Contributions

#N.H. and L.S. contributed equally.

Notes

The authors declare no competing financial interest.

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