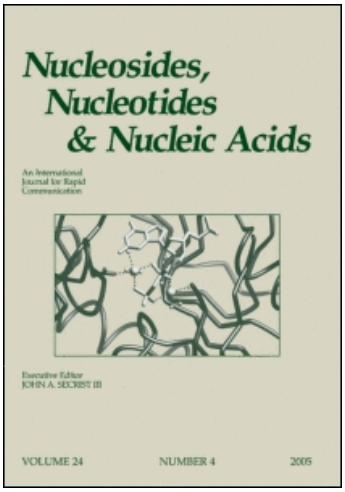

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LIVER TARGETING OF HEPATITIS-B ANTIVIRAL LAMIVUDINE USING THE HEPDIRECT™ PRODRUG TECHNOLOGY

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▫ *A new class of phosphate and phosphonate prodrugs, called HepDirect™ prodrugs, has been developed to deliver drugs to the liver while simultaneously diminishing drug exposure to extra-hepatic tissues. The technology combines liver-selective cleavage and kinase by pass with high plasma and tissue stability to achieve increased drug levels in the liver. Lamivudine (LMV), a nucleoside analogue, is a currently approved treatment for hepatitis B infection, but shows modest efficacy and significant drug resistance due to inefficient phosphorylation. LMV is inadequately phosphorylated to the corresponding nucleoside triphosphate in rat and human hepatocytes. A HepDirect prodrug of LMV monophosphate generated 34-fold higher levels of the triphosphate in rat hepatocytes and 320-fold higher triphosphate levels in the liver of treated rats relative to LMV.*

INTRODUCTION

Hepatitis B virus (HBV) infection is a serious global health problem with 2 billion people infected worldwide, and 350 million suffering from chronic HBV infection. HBV infections result in 500,000 to 1.2 million deaths per year.[1] The three currently approved treatments for HBV, interferon-α, lamivudine (LMV), and adefovir dipivoxil, are inadequate. Interferon-α has a treatment benefit in only approximately 20% of patients,[2] LMV therapy is associated with modest efficacy (2.5–3 log HBV DNA reduction)[3] and significant drug resistance,[4] while the therapeutic potential of adefovir dipivoxil[5] is limited by renal toxicity.[6]

The modest efficacy of LMV and associated drug resistance may be partially explained by the poor conversion of LMV to the corresponding triphosphate. Phosphorylation of LMV to the triphosphate (LMV-TP) is a requirement for the inhibition of the target enzyme, HBV DNA polymerase.[7] L-Nucleoside analogs like LMV are known to be poor substrates for deoxycytidine kinase, the enzyme believed to be responsible for the conversion of LMV to its monophosphate.

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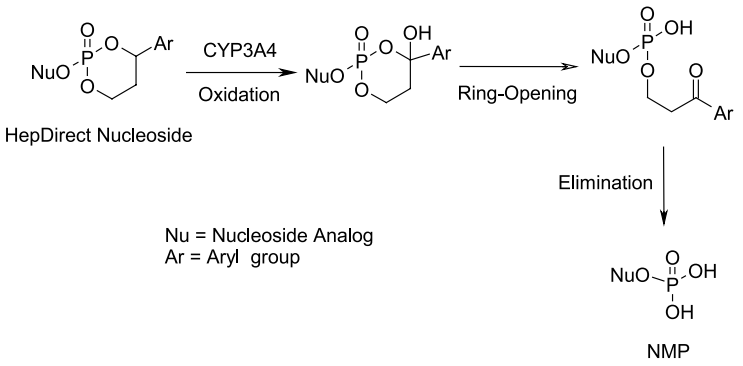


FIGURE 1 Activation of HepDirect prodrugs.

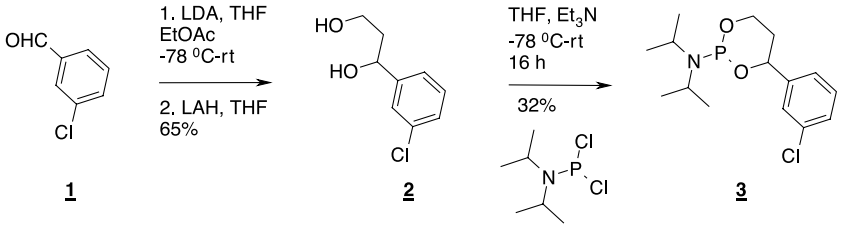
HepDirect™ prodrugs represent a new class of phosphate and phosphonate prodrugs that selectively deliver drugs to the liver.[8] HepDirect prodrugs are resistant to esterase cleavage and therefore are stable in blood and most tissues. In the liver, HepDirect prodrugs undergo oxidative cleavage by the cytochrome P450 isoenzyme CYP3A to produce the corresponding nucleoside monophosphate (NMP), which is rapidly converted to the active nucleoside triphosphate (NTP) by nucleotide kinases.[9] Activation of the HepDirect prodrugs thus bypasses the need for the initial, oftentimes rate-limiting, phosphorylation of nucleosides to NMPs. The objective of the work described herein was to apply the HepDirect prodrug technology to LMV and thereby improve its conversion to LMV-TP in liver and provide a more efficacious therapy for HBV.

PRODRUG DESIGN AND MECHANISM OF ACTIVATION

HepDirect prodrugs are cyclic-1,3-propanyl esters substituted with groups that promote an oxidative cleavage reaction. Oxidation of the C4-methine via a CYP3A-catalyzed reaction results in ring-opening to produce an intermediate that undergoes a beta-elimination to the NMP. The NMP subsequently undergoes further phosphorylation by nucleotide kinases to form the active drug metabolites (Figure 1). The resulting aryl vinyl ketone byproduct undergoes conjugation with liver-abundant glutathione (GSH).

SYNTHESIS

Synthesis of the LMV HepDirect prodrug containing a 4-aryl substituent started from the highly reactive P(III) reagent **3**. 1-Aryl-propane-1,3-diol **2** was synthesized via an aldol condensation involving 3-chlorobenzaldehyde and the enolate of ethyl acetate generated by LDA. The resulting β-hydroxy ester[12] was reduced with LAH to yield 3(3-chlorophenyl)-propane-1,3-diol (**2**). Commercially available di-isopropylphosphoramidous dichloride was reacted with diol **2** in the presence of



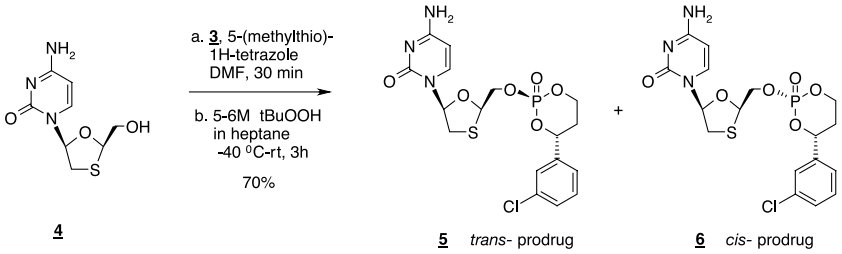
SCHEME 1

triethylamine to give a stable 6-membered ring phosphoramidite **3**. The phosphoramidite was purified by a quick filtration through a silica gel column and was distilled under vacuum to give pure **3** as a low melting solid.

LMV[13] (**4**) was reacted with phosphoramidite **3** in the presence of 5-(methylthio)-1H-tetrazole in DMF to result in the corresponding phosphite.[14] The intermediate phosphite was then oxidized in situ using a 5-6M ^{t}BuOOH solution in heptane at low temperature (−40°C) to avoid decomposition from the resulting exothermic oxidation reaction to give a diastereomeric mixture of oxidized products (**5** and **6**).[15] Diastereomers at the phosphorus center were isolated by column chromatography to give 70% overall yield of **5** and **6** from **4**. The stereochemistry of **5** and **6** was assigned based on the known 6-membered phosphorinane ring systems[16,17] and NOE studies on similar prodrugs. The diastereomer with the 2- and 4-substituents on different sides of the ring plane is called the *trans* isomer (**5**) and the diastereomer with the 2- and 4-substituents on the same side is called the *cis*isomer (**6**).

METHODS

The kinetics of activation of **5** and **6** by CYP3A were evaluated in pooled human and rat liver microsomes as measured by the GSH byproduct capture assay previously described.[8] For hepatocyte activation studies, **4**, **5** and **6** were incubated with freshly isolated rat hepatocytes (n = 2) and intracellular levels of LMV-TP were determined.[8,9] To evaluate the liver targeting potential of **6**, male Simonsen albino (S.A.) rats were administered an IV bolus of either 230 mg/kg



SCHEME 2

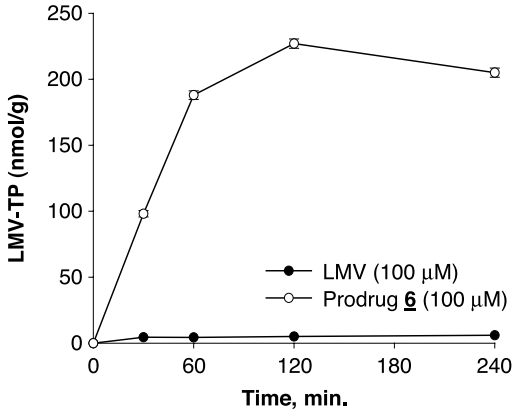


FIGURE 2 LMV-TP generation in rat hepatocytes.

LMV (**4**) or HepDirect-LMV (**6**) (30 mg/kg LMV molar equivalents). At 0.33, 1, 3, 6, 12, and 24 hr following dosing, blood and liver were collected under anesthesia ($n = 4$ rats/time point/test compound), processed, and subjected to HPLC analysis. Blood samples were analyzed for LMV (**4**) and liver samples analyzed for LMV-TP.[8,9]

RESULTS

Activation of *5* and 6 in Rat and Human Liver Microsomes

The mean (±½ range) K_m and V_{max} values for the activation of **6** in pooled mixed human liver microsomes were 73.3 ± 13 μM and 0.19 ± 0.01 nmol/min/mg, respectively. The intrinsic clearance value was 2.6 ± 0.6 μL/min/mg. The corresponding mean (±s.d.) K_m, V_{max}, and CL_{int} values in pooled male rat liver microsomes were calculated to be 63.9 ± 3 μM, 0.51 ± 0.03 nmol/min/mg, and 8.0 ± 0.4 μL/min/mg, respectively. Activation of **5** was undetectable, indicating a microsomal enzyme preference for the *cis*-diastereomer **6**.

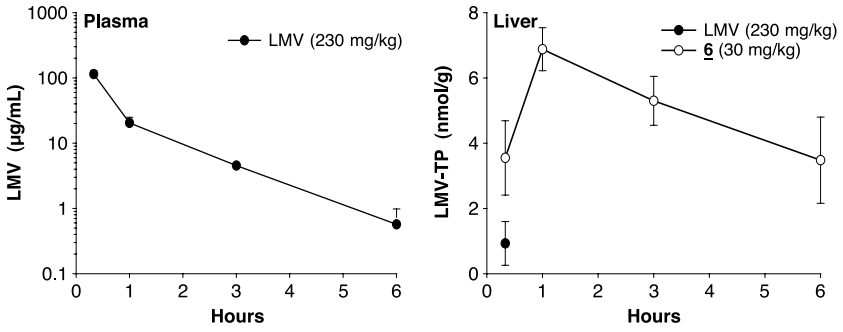


FIGURE 3 Liver targeting of LMV vs. Prodrug 6 in the rat.

TABLE 1 AUCs of LMV and LMV-TP in Rat Liver and Plasma

Test compound	Liver NTP (nmol*g/h)	Plasma LMV, (μM*h)
LMV (**4**), 230 mg/kg	2.6	617
Prodrug (**6**), 30 mg/kg	29.4	<12.8

Activation of 6 in Isolated Rat Hepatocytes

The concentration-time profiles of LMV-TP levels following incubation of freshly isolated rat hepatocytes with 100 μM of **6** and LMV (**4**) are shown in Figure 2. The AUC_{0-4h} (mean ± half range) of LMV-TP was 648 ± 88 nmol*h/g for **6** compared to 18.9 ± 0.3 nmol*h/g for LMV (**4**). In accordance with the microsomal activation data, LMV-TP formation was not seen with diastereomer **5**.

Liver Targeting of 6 versus LMV (4) in the Rat

The temporal profile of LMV **4** (230 mg/kg) and **6** (30 mg/kg, LMV molar equivalents) in plasma and liver following IV administration to rats is shown in Figure 3. Despite high levels of nucleoside in plasma following administration of LMV (**4**), LMV-TP was evident in liver at low concentrations at a single time point only (0.9 nmoles/g at 20 min). Rats administered **6**, in contrast, showed no detectable LMV (**4**) in plasma and significant LMV-TP generation in liver at all time points examined. The mean (± s.d.) C_{max} and half-life values of LMV-TP in liver were 6.9 ± 0 and ~5 h, respectively, following administration of **6**. The AUCs of LMV (**4**) in plasma and LMV-TP in liver for both test compounds are shown in Table 1. Based on the AUC values, the liver-targeting index (liver LMV-TP–to-plasma LMV) of **6** and LMV (**4**) was calculated to be > 2.3 and 0.007, respectively. This represents a >320-fold enhancement in liver targeting of **6** relative to LMV (**4**).

DISCUSSION

HepDirect prodrugs achieve liver selectivity by virtue of being activated via the action of a microsomal cytochrome P450 enzyme, CYP3A,[8] which is predominantly expressed in the liver. In humans, the CYP3A isoenzyme responsible for HepDirect prodrug activation, CYP3A4, is largely expressed in the liver, with lower levels in the intestine, stomach, and colon and undetectable levels in other tissues.[18] Accordingly, HepDirect prodrugs are capable of the delivery of NMPs selectively to the liver. Delivery of the NMP is an important characteristic since phosphorylation of nucleosides to their corresponding monophosphates is generally the rate-limiting step in their conversion to NTPs. This is clearly the case for LMV (**4**), which is a poor substrate for nucleoside kinases and consequently results in modest levels of LMV-TP in rat hepatocytes as well as following intravenous administration of LMV at a high dose (230 mg/kg) to rats. In contrast, the HepDirect prodrug of LMV, **6**, generated ~35-fold higher LMV-TP levels in rat hepatocytes

versus an equivalent test concentration of LMV (**4**), and >10-fold higher liver LMV-TP levels in rats versus a 7.6-fold higher molar equivalent dose of LMV (**4**). The enhanced NTP generation by **6** is likely a reflection of its efficient microsomal activation in hepatocytes to generate high levels of intracellular LMV-MP. The latter bypasses the slow nucleoside kinase step that limits the conversion of LMV (**4**) to LMV-MP and ultimately to the active antiviral metabolite, LMV-TP.

Another important finding from this study was that following administration of **6** to rats and liver-specific mechanism of activation, there was no detectable LMV in plasma. While toxicity of systemically circulating LMV is not a major concern, nucleoside exposure to the periphery is a potential issue for other therapeutic agents. The therapeutic potential of adefovir, for instance, is limited by extrahepatic exposure that leads to renal toxicity.[6] Whether a liver-targeted HepDirect prodrug will significantly improve the therapeutic window for this antiviral nucleoside is currently being tested clinically.[19]

In summary, the favorable cellular and in vivo profile of **6** relative to LMV (**4**), supports the potential clinical utility of a HepDirect prodrug of LMV. Enhanced hepatic LMV-NTP formation by means of the HepDirect prodrug strategy is likely to confer more rapid and complete suppression of HBV replication and decrease the emergence of drug-resistant HBV strains, a major problem with LMV and other nucleoside-based therapies.

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20. **Phosphoramidite** (**3**): R_f = 0.50 in 5% EtOAc in hexanes. mp 44–46°C. ^{1}HNMR (200Mz, DMSO-d_6) 7.5–7.2 (m, 5H), 5.2–5.0 (m, 1H), 4.2–4.0(m, 2H), 3.9–3.6 (m, 2H), 1.8–1.5 (m, 2H), 1.3–1.0 (12H, series of d). ***trans-isomer*** (**5**): R_f = 0.45 (10% MeOH in CH_2Cl_2). ^{1}H NMR (200Hz, DMSO-d_6): 7.80–7.60 (m, 2 H), 7.52–7.16 (m, 4 H), 6.32–6.16 (m, 1 H), 5.80–5.36 (m, 4 H), 4.56–4.20 (m, 4 H), 3.46–3.22 (m, 1 H), 3.18–2.98 (m, 1 H), 2.26–2.00 (m, 2 H). Anal. calcd for $C_{17}H_{19}ClN_3O_6PS$. 0.5 H_2O. 0.1 CH_2Cl_2: C, 42.82 H, 4.25 N, 8.74. Found: C, 42.71 H, 3.82 N, 8.54. ***cis-isomer*** (**6**): mp 110–113°C. R_f = 0.40 (10% MeOH in CH_2Cl_2). ^{1}H NMR (200Hz, DMSO-d_6): 7.66 (s, 1 H), 7.62 (s, 1 H), 7.50–7.21 (m, 4 H), 6.22 (t, J = 6 Hz, 1 H), 5.78–5.62 (m, 3 H), 5.42–5.30 (m, 1 H), 4.62–4.20 (m, 4 H), 3.45–3.24 (m, 1 H), 3.18–2.97 (m, 1 H), 2.40–2.05 (m, 2 H). Anal. calcd for $C_{17}H_{19}ClN_3O_6PS$. 0.75H_2O: C, 43.14 H, 4.37 N, 8.88. Found: C, 43.00 H, 3.95 N, 8.70.