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Design, Synthesis, and Characterization of a Series of Cytochrome P_{450} 3A-Activated Prodrugs (HepDirect Prodrugs) Useful for Targeting Phosph(on)ate-Based Drugs to the Liver§

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Abstract: A new class of phosphate and phosphonate prodrugs, called HepDirect prodrugs, is described that combines properties of rapid liver cleavage with high plasma and tissue stability to achieve increased drug levels in the liver. The prodrugs are substituted cyclic 1,3-propanyl esters designed to undergo an oxidative cleavage reaction catalyzed by a cytochrome P_{450} (CYP) expressed predominantly in the liver. Reported herein is the discovery of a prodrug series containing an aryl substituent at C4 and its use for the delivery of nucleoside-based drugs to the liver. Prodrugs of 5′-monophosphates of vidarabine, lamivudine (3TC), and cytarabine as well as the phosphonic acid adefovir were shown to cleave following exposure to liver homogenates and exhibit good stability in blood and other tissues. Prodrug cleavage required the presence of the aryl group in the *cis*-configuration, but was relatively independent of the nucleoside and absolute stereochemistry at C4. Mechanistic studies suggested that prodrug cleavage proceeded via an initial CYP3A-catalyzed oxidation to an intermediate ring-opened monoacid, which subsequently was converted to the phosph(on)ate and an aryl vinyl ketone by a β-elimination reaction. Studies in primary rat hepatocytes and normal rats comparing 3TC and the corresponding HepDirect prodrug demonstrated the ability of these prodrugs to effectively bypass the rate-limiting nucleoside kinase step and produce higher levels of the biologically active nucleoside triphosphate.

Introduction

Nucleosides are a well-studied class of drugs useful for treating viral infections[1] and leukemia.[2] In most cases, inhibition of viral replication and cell proliferation is associated with intracellular conversion of nucleosides to nucleoside triphosphates (NTPs), which act either as inhibitors of viral and cellular DNA and RNA polymerases or as chain terminators following incorporation into a growing DNA or RNA strand. Because many NTPs are associated with poor polymerase specificity and substantial toxicity, efforts over the past two decades have primarily focused on the discovery of more potent and safe nucleoside analogues through modification of the core nucleoside structure.[3] Some of these modifications have led to NTPs with high polymerase specificity and increased inhibitory potency. Unfortunately, many of the structural modifications have simultaneously compromised NTP production.[4] Typically the poor conversion is attributed to the narrow substrate specificity of the nucleoside kinase[5] and its inability to catalyze the initial phosphorylation, that is, conversion of the nucleoside to the nucleoside 5′-monophosphate (NMP). Subsequent phosphorylations are catalyzed by nucleotide kinases, which generally exhibit a greater tolerance for structural modifications and are not usually rate-limiting.

One potential strategy for increasing NTP production uses cell permeable NMP prodrugs to bypass the nucleoside kinase (Figure 1).[6] Prodrugs from different classes, including acyloxyalkyl esters[7] (**1**), S-acyl-2-thioethyl (SATE) esters[8] (**2**), and more recently a series of phosphoramidates[9] (**3**), are reported to increase NTP levels in nucleoside kinase-deficient cell lines

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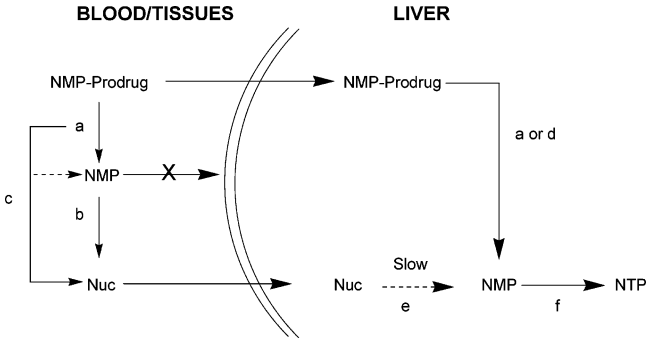
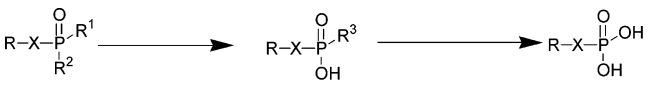


Figure 1. Pathways and enzymes catalyzing nucleoside monophosphate (NMP) prodrug cleavage and conversion to corresponding nucleoside (Nuc) and nucleoside triphosphate (NTP): (a) esterase or esterase and phosphoramidase; (b) phosphatase; (c) phosphodiesterase (cleaves monoacid); (d) CYP 3A; (e) nucleoside kinase; (f) nucleotide kinase(s).

Scheme 1



X = O or CH_2

$R^3 = R^2$ or metabolite of R^2

1 $R^1 = R^2 = OCH_2OC(O)R^4$
2 $R^1 = R^2 = OCH_2CH_2SC(O)R^4$
3 $R^1 = OC_6H_5$, $R^2 = N(H)CH(CH_3)COOEt$
4 $R^1 = OH$, $R^2 = OCH_2CHO[C(O)R^4]CH_2OC(O)R^4$
5 $R^1 = R^2 = OC_6H_5$
6 $R^1 = R^2 = OCH_2C_6H_4(4\text{-}OCH_3)$

(Scheme 1). While promising, use of these prodrugs for bypassing the kinase in vivo is compromised by the dependence of prodrug cleavage on esterases and consequently their rapid breakdown in blood and nearly all tissues in the body. Prodrug cleavage outside of the target organ limits NTP production, because the initial cleavage produces an intermediate mono acid, which is poorly transported across cell membranes and undergoes further breakdown to the corresponding nucleoside via phosphodiesterases (PDEs) present in the blood or tissues or via phosphatases following breakdown of the intermediate monoacid to the NMP.

An alternative kinase bypass strategy is to replace the phosphate group of the NMP with a phosphonic acid,[10] because phosphonic acids are resistant to both phosphodiesterases and phosphatases. In a few cases, the modification results in compounds which are able to undergo further phosphorylation to the diphosphate (NTP mimetic) and inhibition of the targeted polymerase. The high negative charge on phosphonic acids, however, limits transport across cell membranes and oral bioavailability. Esterase-sensitive prodrugs improve oral bioavailability, but are often of limited value for delivery of phosphonic acids to selective tissues due to rapid and widespread prodrug breakdown in blood and nontarget tissues.

Nonesterase-sensitive prodrugs represent an attractive alternative strategy if the enzyme(s) catalyzing prodrug cleavage is expressed in the target tissue and is relatively efficient. For example, phospholipid prodrugs of NMPs (**4**) are reported to cleave by a two-step process involving phospholipase A and lysophospholipase and produce modestly higher NTP levels in the liver relative to the corresponding nucleoside.[11] Diphenyl esters (**5**),[12] substituted dibenzyl esters (e.g., **6**),[13] and certain alkyl esters[6] cleave by nonesterase-mediated reactions that result in rapid cleavage of the first ester to produce an intermediate monoacid. The second cleavage step, however, is often very slow, greatly diminishing the value of these prodrugs for NTP production, especially as applied to phosphates, which as the monoacid are simultaneously converted to the nucleoside by PDEs.

Prodrug Design. In an effort to discover new drugs for treating liver diseases such as hepatitis B (HBV), hepatitis C (HCV), and hepatocellular carcinoma (HCC), a prodrug strategy was sought that could deliver NMPs to the liver in high concentrations and with high liver specificity. The prodrug characteristics needed to achieve this goal included: (1) rapid activation by an enzyme expressed predominantly in the liver and efficient conversion to the NMP using only one enzyme-catalyzed reaction; (2) good stability in aqueous solutions, blood, and nonhepatic tissues; and (3) no byproduct-related toxicity.

To achieve these goals, our efforts focused on cyclic 1,3-propanyl esters with the knowledge that two marketed phosphoramide prodrugs, cyclophosphamide (CPA, **7**) and ifosfamide (IFA, **8**),[14] cleave to anticancer agents following a cytochrome P_{450} (CYP)-catalyzed hydroxylation (Figure 2).[15] The hydroxylated product (**9**) ring opens to form **10**, which subsequently undergoes a β-elimination reaction. Because the equilibrium governing ring opening favors **9**, the β-elimination reaction is relatively slow, allowing time for **9** to escape the liver and distribute into tissues. Accordingly, while activated in the liver, both CPA and IFA are used to treat a variety of solid tumors outside of the liver. Efforts to apply the analogous prodrug strategy to the NMP of 2′-deoxyfloxuridine (FUdR) (**11**) failed to result in ring oxidation, prodrug cleavage, or evidence for oncolytic activity in vivo.[16] Similarly, Starrett reported that an analogous ester of a phosphonic acid also failed to cleave.[17] Cleavage was achieved when the ring was substi-

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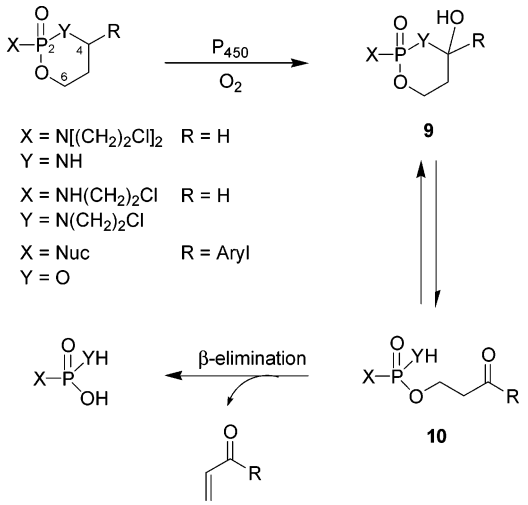
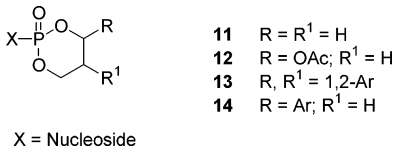


Figure 2. Prodrug cleavage mechanism.

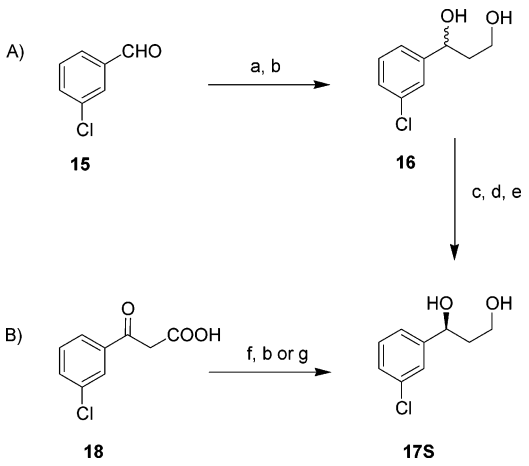
Scheme 2



tuted at C4 with an OAc (**12**)[18] or when an aryl was fused to the ring (**13**) (Scheme 2). Neither prodrug, however, represents a satisfactory solution, because **12** cleaves using an esterase and **13** cleaves via a tandem two-step chemical cleavage[19] that requires balancing the need for rapid hydrolysis in vivo to produce adequate NTP levels with a need for long-term hydrolytic stability for drug manufacture and storage.

On the basis of these results and knowledge of various CYP properties,[20] including tissue distribution and substrate SAR, we initiated work on a strategy that uses substituents on the ring to promote an oxidative cleavage reaction. Discovery of esters with these properties led to studies demonstrating the potential of this new class of prodrugs, which we call HepDirect, to deliver certain drugs to the liver and achieve improved efficacy and/or safety. Reported herein is the synthesis and characterization of the first HepDirect prodrug series, a series in which the substituent is a C4 aryl group (**14**). The series was designed on the basis of the postulate that a benzylic carbon would make the prodrug more susceptible to CYP-mediated oxidation and that, unlike CPA and IFA, oxidation of **14** would produce a negatively charged, ring-opened intermediate (**10**), which could result in increased cellular retention if the negative charge slowed export of **10** out of the cell and if ring closure to **9** was an unfavorable process. Application of the HepDirect prodrug strategy to NMPs led to increased NTP levels in hepatocytes and in the livers of treated rats.

Scheme 3 [a]



[a] Conditions: (a) EtOAc, LDA, THF, −78 °C to room temperature; (b) LAH, Et_2O; (c) HMDS, cat. TMSOTf; (d) (−)-menthone, cat. TMSOTf; (e) HCl, MeOH; (f) (−)-DIP−Cl, −20 to 0 °C; (g) BH_3·THF, 0 °C to room temperature.

Results

Prodrug Synthesis. HepDirect prodrugs were synthesized using a convergent synthetic strategy in which suitably protected phosphonic acids and nucleosides were coupled with 1-aryl 1,3-propanediols or related phosphorus-containing intermediates. The racemic diol **16** and the corresponding enantiomers, **17*S*** and **17*R***, were synthesized as shown in Scheme 3. High optical purity was achieved initially through chromatographic separation of diastereomeric (−)-menthone ketals.[21] Alternatively, the single enantiomers of **17** (>96% ee) were prepared by an asymmetric reduction of the aryl ketoacid **18** with (−)- or (+)-DIP chloride[22] followed by reduction of the resulting β-hydroxy acid with LAH or BH_3·THF.

Prodrugs of adefovir (PMEA) were initially prepared by heating a mixture of PMEA, **16**, DCC, and pyridine in DMF at 100 °C for 12−16 h. The product mixture contained both the *cis* (**21**) and the *trans* (**22**) racemic diastereomers, which were readily separated using chromatography and fractional crystallization. Structures were assigned on the basis of ^{1}H and ^{31}P NMR chemical shifts reported for other cyclic phosph(on)ate esters.[23] The X-ray structure of **19** subsequently confirmed the assignments (data not shown). Neither the monoacid nor the diester were observed in the product mixture in significant quantities, indicating that ring closure to form the cyclic ester was a much more favorable process than reaction with a second diol equivalent.

The *cis*:*trans* ratio for PMEA prodrugs produced using the DCC reaction ranged from 55:45 to 0:100. In an effort to increase the preference for the *cis* diastereomer (vida infra), alternative coupling procedures and conditions were evaluated. Reaction of PMEA with oxalyl chloride and *N*,*N*-diethylformamide simultaneously converted the phosphonic acid to the dichloridate and the $^6NH_2$ group to the diethylaminomethyleneimine (Scheme 4). Protection of the $^6NH_2$ group greatly

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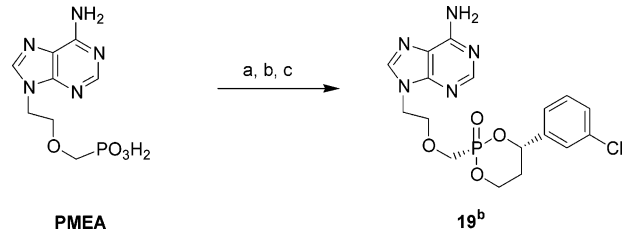
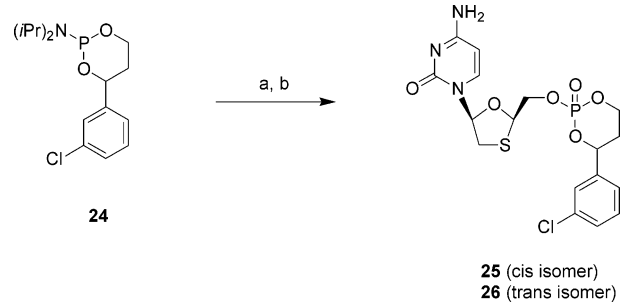
Scheme 4 [a]



PMEA **19**[b]

[a] Conditions: (a) $(COCl)_2$, DEF, reflux; (b) **17*S***, TEA, pyr, −78 to 0 °C; (c) HOAc, reflux. [b]Structure of *cis*-isomer.

Scheme 5 [a]



24

25 (cis isomer)
26 (trans isomer)

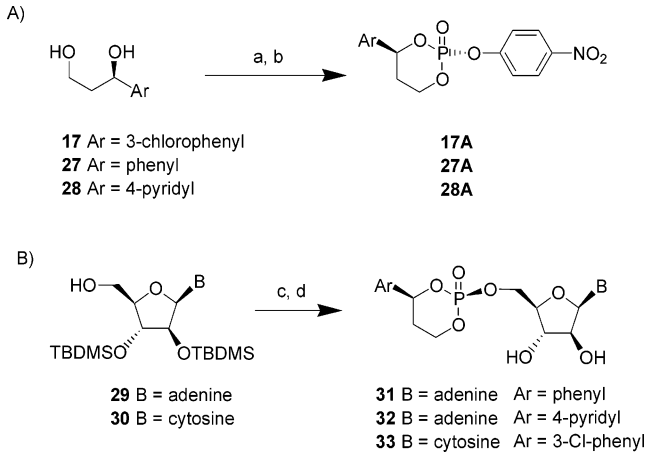
[a] Conditions: (a) 3TC, 5-(methylthio)-1*H*-tetrazole; (b) *t*-BuOOH.

improved solubility, which in turn enabled use of lower temperatures in the coupling reaction. Lower temperatures and inverse addition of the dichloridate to the diol increased the *cis*:*trans* ratio to 75:25. After hydrolysis of the imine with acetic acid, the *cis*-isomer was isolated in >95% de by chromatography.

Prodrugs of nucleoside monophosphates were synthesized by coupling the nucleoside to a P(III) phosphoramidite[24] or a P(V) phosphate[25] intermediate. The prodrug of lamivudine (3TC) was prepared using the phosphoramidite **24**, which was readily generated from racemic diol **16** and diisopropyl phosphoramidous dichloride (Scheme 5). Reaction of **24** with 3TC gave an intermediate phosphite, which was oxidized in situ to produce a 60:40 *cis*:*trans* mixture of the corresponding phosphate esters. Chromatography on silica gel readily gave the racemic *cis* and *trans* prodrugs **25** and **26**. The structure assignments were based on ^{31}P NMR chemical shifts reported for an analogous cyclic phosphate ester.[23]

Prodrugs of vidarabine (araA) and cytarabine (araC) were prepared by an S_N2 reaction between the 5′ hydroxyl of 2′,3′ diprotected nucleosides and a *p*-nitrophenyl phosphate (Scheme 6). High *cis* stereospecificity was achieved by preparing the phosphate intermediate as the *trans*-isomer. Reaction of the 1,3-propanediol (e.g., **17**) with *p*-nitrophenyl phosphorodichloridate gave the cyclic phosphate intermediate (e.g., **17A**) as an approximate 50:50 *cis*:*trans* mixture. Stirring the mixture overnight in the presence of 4 equiv of *p*-nitrophenol and Et_3N equilibrated the phosphates and produced the thermodynamically favored *trans*-isomer (<3% of the *cis*-isomer). Coupling of the phosphate intermediate to protected nucleosides proved to be the most challenging reaction with numerous conditions resulting in poor yields and stereochemical mixtures. Fortunately, the magnesium salt of the 5′ hydroxyl of nucleosides reacted with

Scheme 6 [a]



17 Ar = 3-chlorophenyl
27 Ar = phenyl
28 Ar = 4-pyridyl

17A
27A
28A

29 B = adenine
30 B = cytosine

31 B = adenine Ar = phenyl
32 B = adenine Ar = 4-pyridyl
33 B = cytosine Ar = 3-Cl-phenyl

[a] Conditions: (a) 4-nitrophenyl phosphorodichloridate; (b) *p*-nitrophenol, TEA; (c) *tert*-butyl MgCl, and **17A**, **27A**, or **28A**; (d) TEAF or TBAF.

the phosphate intermediate with inversion of configuration to give *cis* prodrugs (<3% *trans*-isomer), which were isolated in 40−60% overall yield following TBDMS deprotection.

HepDirect Prodrug Activation Mechanism. The concentration of 3TC, araA, araC, and PMEA prodrugs incubated in the presence of liver microsomes and NADPH was monitored by HPLC and shown to generate product linearly with time. The rate of conversion was linearly dependent on protein concentration over a range of at least 0.3−3 mg/mL microsomal protein. No conversion was observed in the absence of NADPH or with the cytosolic fraction (<10 pmol/min/(mg of protein)). To determine whether CYPs were responsible for prodrug activation, the PMEA prodrug **21** was incubated with human liver microsomes in the presence of the known CYP inhibitors, furafylline (CYP1 inhibitor), sulfaphenazole (CYP2 inhibitor), and ketoconazole (CYP3A inhibitor). No inhibition was observed with furafylline or sulfaphenazole, whereas ketoconazole inhibited prodrug conversion in a concentration-dependent manner with complete inhibition observed at 10 μM. The ketoconazole IC_{50} was 0.5 μM, which is consistent with the IC_{50}'s reported for ketoconazole and other CYP3A substrates.[20b,d]

CYP isoenzyme specificity was further evaluated against a panel of recombinant human CYP enzymes. Consistent with the studies using CYP inhibitors, prodrug **21** was activated predominantly by CYP3A (Table 1). Within the CYP3A isoenzyme family, activation by CYP3A4 was at least 20-fold higher than activation by either CYP3A5 or CYP3A7. The high CYP3A4 specificity was confirmed in studies showing that the activation rate of **21** was decreased >90% using human microsomes pretreated with a CYP3A4 antibody. Similar CYP3A specificity was observed with other NMP prodrugs, but in some cases with less discrimination between CYP3A4 and CYP3A5.

HPLC and LC/MS analysis of the products generated from incubating prodrugs with rat liver microsomes helped identify the site of oxidation and the mechanism of prodrug cleavage. Incubation of **21** in the presence of 10 mM glutathione gave PMEA and a glutathione conjugate (Scheme 7). The conjugate, which was formed at similar rates in the presence and absence of glutathione S-transferase, was identified as compound **38** on the basis of LC/MS data (MW = 473) and its coelution with a standard prepared by reaction of glutathione and 3-chlorophenyl

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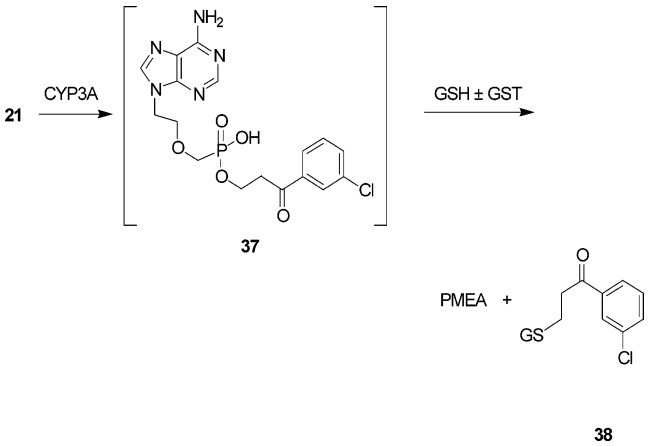
Table 1. Human CYP Isoenzyme Activation of **21**[a]

isoenzyme[b]	rate[c]	% CYP3A4 activity
1A1	0.36 ± 0.04	7
1A2	0.4 ± 0	7
1B1	0.57 ± 0.02	10
2A6	0.54 ± 0.04	10
2B6	0.76 ± 0.16	14
2C8	0.25 ± 0.01	4
2C9	0.42 ± 0.01	8
2C18	0.29 ± 0	5
2C19	0.51 ± 0.12	9
2D6	0.49 ± 0.03	9
2E1 + b5	<0.1	<2
3A4	5.58 ± 0.17	100
3A4 + b5	30.8 ± 0.6	550
3A5	0.27 ± 0.06	5
3A7 + b5	0.99 ± 0.03	20
4A11	0.21 ± 0.04	4

[a] Initial rates determined at 250 μM **21**. [b] Isoenzymes purchased from BD Biosciences as supersomes coexpressing b5 when denoted. Specific activities for each isoenzyme with a known substrate (e.g., CYP3A4 with testosterone is 8.2) are reported in the Supporting Information. [c] pmol/(pmol of CYP)/min ± s.d.

Scheme 7 [a]



[a] Abbreviations: PMEA = 9-(2-phosphonylmethoxyethyl)adenine; GSH = glutathione; GST = glutathione S-transferase.

vinyl ketone. Conversion of **21** to **38** was completely inhibited by ketoconazole and required glutathione in the incubation mixtures to trap the highly reactive vinyl ketone byproduct immediately following its production. Conjugate **38** was produced in nearly equimolar amounts to PMEA (92.4 ± 3.7% of PMEA), suggesting that the C4 benzylic carbon was the predominant site of oxidation. In addition to these products, a transient product was detected using reverse phase HPLC. The peak eluted at 13.7 min, which is intermediate between PMEA (R_t = 10.5 min) and the PMEA prodrug **21** (R_t = 14.2 min). LC/MS analysis of the peak gave a molecular ion with a mass (441 in positive mode) identical to the molecular weight of the corresponding hydroxylated prodrug **9** or ring-opened monoacid **10** (Figure 2). Subsequent studies assigned the structure of the intermediate to the ring-opened compound (**37**) based on its coelution with a synthetic standard and ^{1}H NMR spectra (Scheme 7). Incubation of **37** in aqueous solutions failed to produce a peak in the HPLC chromatogram, consistent with the ring-closed product. Moreover, at pH 7 or higher, **37** underwent a facile base-catalyzed β-elimination reaction to produce PMEA ($t_{1/2}$ = 2.8 h, pH = 9, room temperature). Interestingly, no intermediate was detected when prodrugs of phosphates were incubated with microsomes under similar conditions, possibly suggesting that the β-elimination reaction for the intermediate phosphate is faster than that for phosphonates.

Table 2. SAR of Prodrug **14** Cleavage[a]

cmpd	Nuc	R	Ismr	C4	activity[b]
19	PMEA	3-Cl Phe	*cis*	*S*	510 ± 6
20	PMEA	3-Cl Phe	*cis*	*R*	193 ± 11
21	PMEA	3-Cl Phe	*cis*	*R/S*	421 ± 77
22	PMEA	3-Cl Phe	*trans*	*R/S*	<10
23	PMEA	H			<10[c]
25	3TC	3-Cl Phe	*cis*	*R/S*	101 ± 3
26	3TC	3-Cl Phe	*trans*	*R/S*	<10
31	araA	Phe	*cis*	*S*	102 ± 8[d]
32	araA	4-Pyr	*cis*	*S*	26 ± 2[d]
33	araC	3-Cl Phe	*cis*	*S*	79 ± 14
34	araC	3-Cl Phe	*cis*	*R*	120 ± 23
35	araC	3-Cl Phe	*cis*	*R/S*	133 ± 12
36	araC	3-Cl Phe	*trans*	*R/S*	<10

[a] Initial rates were determined at 250 μM prodrug concentration using pooled human liver microsomes (IVT, lot RQX). [b] pmol/min/(mg of protein) as measured by byproduct capture assay unless otherwise denoted. [c] PMEA production monitored by HPLC. [d] araAMP production monitored by ion exchange HPLC.

Table 3. Kinetic Parameters[a]

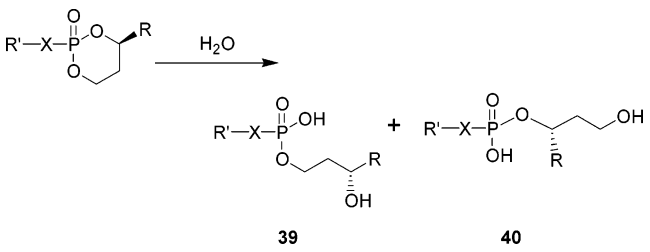
	male rat liver			human liver[b]		
cmpd	K_M	V_{max}	Cl_{int}	K_M	V_{max}	Cl_{int}
19	25 ± 7	1.2 ± 0.1	48.1 ± 8	160 ± 9	1.8 ± 0.03	11.3 ± 0.4
25	63.9 ± 3	0.51 ± 0.0	8.0 ± 0.4	73.1 ± 13	0.19 ± 0.01	2.6 ± 0.6
33	ND	ND	ND	484 ± 112	0.86 ± 0.12	1.9 ± 0.2
34	ND	ND	ND	519 ± 93	0.54 ± 0.01	1.1 ± 0.2
IFA[c]	4300 ± 380	2.58 ± 0.06	0.6 ± 0.04	8285 ± 1445	4.57 ± 0.58	0.56 ± 0.03

[a] Determined using rat and human liver microsomes, units: K_M is μM, V_{max} is nmol/min/(mg of protein), Cl_{int} is μL/min/(mg of protein). [b] Pooled human liver microsomes (IVT lot #1037). [c] Literature values for IFA (**8**) with rat microsomes are K_M = 2400 ± 230; V_{max} = 3.04 ± 0.26; Cl_{int} = 1.27 (ref 35).

Prodrug Cleavage Kinetics and SAR. The structural features influencing prodrug activation were derived from the kinetic results obtained from 13 prodrugs (Table 2). Facile prodrug cleavage required the presence of a C4 aryl group based on the lack of detectable activation of the unsubstituted ester of PMEA (**23**) (<10 pmol/min/mg) and the >50-fold higher activation rate observed for the 3-chlorophenyl-substituted prodrug **19**. Prodrug cleavage rates were modestly sensitive to the nucleoside based on the less than 10-fold difference in rates for NMP analogues of araC, araA, and 3TC. A similar narrow range of specific activities was observed for prodrugs with different aryl groups (phenyl, 3-chlorophenyl, and 4-pyridyl). Prodrug activation was also relatively insensitive to the absolute stereochemistry at C4 based on the approximate 2-fold difference observed for the corresponding prodrugs of PMEA and araCMP. In contrast, prodrug conversion was highly dependent on the relative stereochemistry between C4 and the phosphate with conversion only observed for the *cis*-configuration, that is, the isomer in which the aryl and nucleoside groups are in a cis relationship to each other.

Kinetic parameters were determined for prodrugs of PMEA (**19**), 3TC-MP (**25**), and araCMP (**33** and **34**) using microsomes isolated from rat and human livers (Table 3). K_M's for the prodrugs ranged between 73 and 519 μM. Catalytic efficiencies or intrinsic clearance rates (V_{max}/K_M) were equivalent to or better

Scheme 8



than IFA (**8**), an oncolytic drug extensively metabolized in humans by CYP3A4.[14c,20d]

Prodrug Stability and Solubility. HepDirect prodrug stability in aqueous solutions was surprisingly high given the poor stabilities reported for benzyl esters[13] and other cyclic phosphate[26] and phosphonate esters.[17] As expected for ester hydrolysis, the hydrolytic rate was highly dependent on pH with hydrolysis readily observed above pH 9 ($t_{1/2}$ = 11.6 h, pH 9) and to a lesser extent at low pH ($t_{1/2}$ > 7 days at pH 1). At pH 7, the t_{90} (time to 90% of starting amount) for **21** was >3 days. Good aqueous stability was also observed for prodrugs of phosphates; for example, the t_{90} for **35** was >6 days at pH 7. In some cases, the aryl group influenced prodrug stability, solubility, or both. For example, the araAMP prodrug with a C4 phenyl (**31**) had a t_{90} (pH 7) of ~12 h and a solubility of 0.38 mg/mL, whereas the araAMP prodrug with a C4 pyridyl (**32**) had a t_{90} (pH 7) of >120 h and a solubility of 1.5 mg/mL. Interestingly, hydrolysis of prodrug **21** gave both ring-opened products, that is, **39** and **40** (Scheme 8), in approximately equal amounts, suggesting that hydrolysis proceeded through a mechanism independent of the aryl group.

Prodrug stability was also evaluated in blood and various rat tissue homogenates, including heart, muscle, lung, brain, kidney, spleen, stomach, testes, intestine, and liver. Only incubations with liver and to a lesser extent intestine (~50% of liver rate per mg of protein) homogenates showed detectable prodrug cleavage, a result consistent with the CYP activation mechanism and the reported tissue distribution of CYP3A.[20b,e]

Hepatocyte Studies. Freshly isolated rat hepatocytes were used to assess cellular penetration and activation of HepDirect prodrugs as well as the metabolic fate of the prodrug cleavage products. Similar studies using either cultured rat hepatocytes or human hepatoma cell lines were not possible due to the lack of appreciable CYP3A activity (≪1%) in cultured cells.[20c] Incubation of **25** with primary rat hepatocytes resulted in concentration- and time-dependent production of 3TC triphosphate (Figure 3). Peak NTP levels and NTP exposure (AUC_{0-4h}) were 33- and 34-fold higher than that found for 3TC-treated hepatocytes (Table 4). Similar improvements were observed with araCMP and PMEA prodrugs but not with the araAMP prodrug **32** primarily because araA is a substrate for adenosine kinase and therefore is readily phosphorylated in hepatocytes. Ketoconazole inhibited the conversion of prodrugs **21** and **35** to the corresponding NTP in rat hepatocytes (IC_{50} of 0.5 ± 0.2 and 0.2 ± 0.1 μM, respectively), consistent with a CYP3A-prodrug cleavage mechanism.

Rat Studies. Prodrug **25** was administered (60.2 mg/kg, iv, 30 mg/kg of 3TC equivalents) to normal overnight fasted rats,

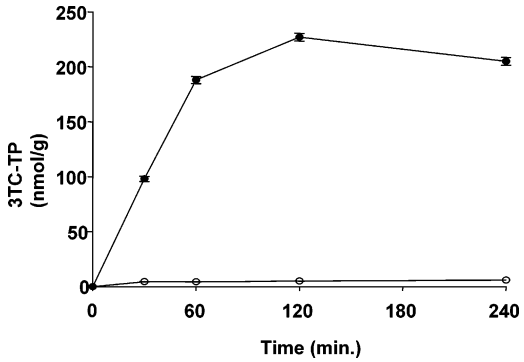


Figure 3. Conversion of 100 μM lamivudine (○) and prodrug **25** (●) to lamivudine triphosphate (3TC-TP) in freshly isolated rat hepatocytes. 3TC-TP AUC_{0-4h} values for lamivudine and **25** were 18.9 and 648 nmol·h/g cells, respectively.

Table 4. Prodrug Activation in Rat Hepatocytes[a]

drug	C_{max} (pmol/mg)	AUC_{0-4h} (pmol·h/mg)
PMEA	41 ± 6	87 ± 12
19	379 ± 131	756 ± 291
3TC	6 ± 0	19 ± 0.3
25	201 ± 35	648 ± 88
araA	379 ± 17	486 ± 3
32	93 ± 13	122 ± 11
araC	<2	<5
35	175 ± 64	398 ± 112

[a] Peak NTP levels and overall exposure were determined for suspended freshly isolated hepatocytes from male rats incubated with drug (100 μM) for 4 h.

and the timecourse of liver 3TC triphosphate and plasma 3TC was compared to that of rats treated with 3TC (230 mg/kg, iv) (Figure 4). Despite the 7.7-fold lower dose (3TC equivalents), **25** produced 7.7-fold higher peak triphosphate levels and an 11.3-fold greater liver exposure (AUC_{0-6h}). The fold increase in AUC represents a lower limit, because the AUC for the 3TC-treated animals was calculated using the limit of quantification (LOQ) (1 nmol/g) for all timepoints associated with undetectable NTP levels (>20 min). Conversely, plasma 3TC was undetected (LOQ = 0.5 μg/mL) in rats treated with **25**, whereas 3TC-treated animals exhibited peak levels of 103 ± 2 μM (20 min) and an AUC_{0-6h} of 617 μM·h (Figure 4B). These results translate to a liver targeting index (AUC_{0-6h} NTP (liver)/AUC_{0-6h} Nuc (plasma)) of >2.3 for **25** and an index of 0.004 for 3TC. The increase in the liver targeting index for **25** is therefore >575-fold.

Discussion

Efficient cleavage of 1,3-cyclic propyl esters of phosphates and phosphonates containing a C4 aryl substituent was observed in vitro using liver microsomes and hepatocytes from rats and humans as well as in vivo. Prodrug cleavage was initiated by a CYP-catalyzed oxidation of C4, which required the presence of an aryl group and the *cis*-configuration (Table 2). The importance of the aryl group for C4 oxidation was attributed to its enhancement of C4 hydrogen abstraction and the corresponding increased susceptibility of benzylic hydrogens to CYP-catalyzed oxidation.[20d] Cleavage rates were less dependent on the absolute configuration at C4. Moreover, cleavage occurred readily for prodrugs of structurally different NMPs, including both purine (araA and PMEA)- and pyrimidine (3TC and araC)-

(26) Khorana, H. G.; Tener, G. M.; Wright, R. S.; Moffat, J. G. *J. Am. Chem. Soc.* **1957**, *79*, 430−436.

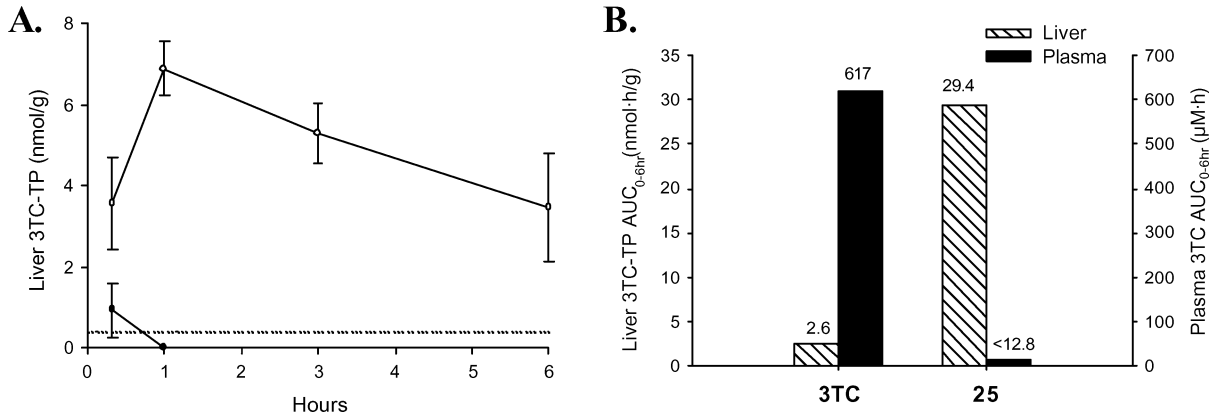


Figure 4. (A) Timecourse of liver 3TC triphosphate (3TC-TP) levels after treating male rats (n = 4/group) with 3TC (●) (230 mg/kg, iv) and prodrug **25** (○) (30 mg/kg of 3TC equivalents, iv). LOQ (dashed line) = 0.5 nmol/g. (B) Liver 3TC-TP AUC_{0-6h} and plasma 3TC AUC_{0-6h} for 3TC and **25**-treated rats.

containing NMPs, as well as NMPs containing various natural and unnatural sugars. These data indicate that the prodrug strategy is applicable to a wide variety of structurally diverse NMPs and phosphonate-containing compounds. Furthermore, the catalytic efficiencies reported in Table 3 are comparable to or better than those of other CYP-metabolized drugs (e.g., IFA),[15,20d] thereby suggesting that prodrugs from this class will undergo extensive cleavage in humans.

The human CYP isoenzyme primarily responsible for catalyzing the oxidative cleavage reaction was identified as CYP3A4. While prodrug cleavage was detected at high prodrug concentration with other human CYPs, cleavage rates were typically 10-fold higher with CYP3A4 (Table 1). Moreover, of the 57 human CYPs[20a] expressed in the liver,[20] most contribute <5% to the total CYP activity, whereas CYP3A4 accounts for approximately 30% of the total.[27] Studies with CYP3A inhibitors and an antibody directed at CYP3A4 confirmed the predominant role of CYP3A4 in the cleavage of this class of prodrugs. These findings suggest that prodrug activation will occur predominantly in the liver, because the liver and to a lesser extent the gastrointestinal tract are the only tissues known to express CYP3A4.[20b,e] Furthermore, unlike other CYPs, appreciable levels of CYP3A4 expression is relatively independent of gender, race, age, and liver disease and is not subject to polymorphism-related differences.[20,27]

The prodrug cleavage mechanism was delineated by analysis of the reaction intermediates and products. CYP3A-catalyzed oxidative cleavage of **21** in the presence of glutathione produced equimolar amounts of PMEA and the glutathione conjugate **38**. These and other findings suggested that CYP3A oxidation occurred exclusively at C4 and that the conjugate was produced via a base-catalyzed β-elimination reaction followed by rapid addition of glutathione to the resulting aryl vinyl ketone. A transient reaction intermediate was also detected and identified as the ring-opened compound **37** (Scheme 7). No additional peaks in the HPLC chromatograms were observed corresponding to the ring-closed product (i.e., **9**, Figure 2), suggesting that the ring-opening reaction was highly favored and essentially irreversible. At pH 7 or higher, **37** underwent a facile β-elimination reaction to produce PMEA. No intermediate was observed with phosphate prodrugs, presumably because of differences in leaving group ability.

These results suggest that prodrug cleavage proceeds by a mechanism similar to that of IFA and CPA,[14,15] but with one important difference. Unlike IFA and CPA, C4 hydroxylation of HepDirect prodrugs favors the ring-opened compound **10** instead of the ring-closed product **9** as the predominant intermediate. The reason for this difference is not entirely clear but is likely related to differences in the stability of the ring-closed forms or more likely differences in the rate of ring closure, which entails addition of a negatively charged phosphonic acid to an aryl ketone instead of the addition of a phosphoramide to an aldehyde. The predominance of **10** over **9** favors product formation because only **10** is capable of undergoing the β-elimination reaction. Furthermore, unlike the reaction intermediates of CPA or IFA, the ring-opened product from HepDirect prodrugs is negatively charged and therefore unable to escape the liver by passive diffusion across the cell membrane. Accordingly, HepDirect prodrugs produce both the product and the prodrug byproduct inside the cell catalyzing the oxidative cleavage reaction.

Confining prodrug cleavage to the liver is expected to greatly improve the therapeutic potential of nucleoside-based drugs for the treatment of liver diseases. Nucleosides, while effective against many viral infections and cancers, have shown only limited success in treating patients with HBV and virtually no success in patients with HCV or primary liver cancer. Much of the failure is attributed to poor efficacy arising from poor conversion of some nucleosides to the NMP and consequently the NTP.[4] In other cases, however, nucleosides fail because they are readily converted to the NTP in tissues outside of the liver which can lead to dose-limiting extrahepatic toxicity.

HepDirect prodrugs of NMPs represent a possible solution to both problems. As demonstrated with prodrugs of several nucleosides, higher NTP levels are achieved in hepatocytes (Table 4) presumably because the prodrugs effectively bypass the rate-limiting kinase responsible for the conversion of nucleosides to the NMP. Furthermore, because prodrug cleavage and NTP production are confined to cells that express CYP3A, HepDirect prodrugs target NTP production to the liver, which reduces the risk of dose-limiting extrahepatic toxicities and may thereby enable higher doses to be administered.

The benefits of the HepDirect prodrug strategy were demonstrated by administering the 3TC prodrug **25** to rats. Relative to rats treated with 3TC, rats treated with **25** at a 7.7-fold lower 3TC equivalent dose showed 7.7-fold higher 3TC-TP peak liver

(27) Shimada, T.; Yamazaki, H.; Mimura, M.; Inui, Y.; Guengerich, F. P. *J. Pharmacol. Exp. Ther.* **1994**, *270*, 414−423.

levels and an 11.3-fold greater liver exposure (AUC_{0-6h}) (Figure 4A). Moreover, rats treated with **25** showed no detectable plasma levels of 3TC (Figure 4B), suggesting that the NMP generated after prodrug cleavage is converted to the NTP and not rapidly dephosphorylated to 3TC by intracellular phosphatases. The absence of detectable plasma 3TC levels also ruled out the possibility that prodrug cleavage intermediates were secreted into the circulation, because **10** would be instantaneously degraded by cellular and plasma PDEs to 3TC.

The pharmacokinetic profile of HepDirect prodrugs differs not only from the profile of the corresponding nucleoside, but also from the expected profile for previously described NMP prodrugs. Most NMP prodrugs are cleaved by esterases and/or other enzymes ubiquitously distributed throughout the body. Rapid prodrug cleavage by esterases in organs outside of the liver diminishes liver NTP production by limiting prodrug exposure to the liver. Moreover, prodrugs cleaved by esterases are rapidly converted in plasma to the corresponding NMPs, which are instantaneously dephosphorylated to the nucleoside by plasma phosphatases. Similarly, prodrugs that undergo conversion to a long-lived intermediate monoacid (Scheme 1) can also generate high levels of the nucleoside in plasma through the action of plasma and tissue PDEs. The high plasma stability of HepDirect prodrugs limits plasma nucleoside levels and therefore diminishes the risk of nucleoside-associated toxicities, which range from various neuropathies (araA, ddI) to myelosuppression (araC, fludarabine, floxuridine) and renal damage (PMEA).[1,2]

Another anticipated benefit of confining prodrug cleavage to CYP3A-containing cells is that it localizes production of the prodrug byproduct, a potentially toxic aryl vinyl ketone,[28] to these cells. Because cells expressing CYP3A also express high levels of glutathione (>5 mM) as a natural defense mechanism against damage from the free radicals generated during CYP-mediated oxidations,[29] the aryl vinyl ketone is expected to be rapidly and quantitatively captured as the glutathione conjugate (e.g., **38**). As was found for the vinyl ketone generated in the liver following CYP2E1-catalyzed oxidation of acetaminophen,[30] intracellular capture of the byproduct and excretion of the conjugate prevents both protein and DNA alkylation and the subsequent cytotoxicity and genetic toxicity commonly associated with vinyl ketones.[31]

Summary

Inadequate efficacy and dose-limiting side effects commonly limit the therapeutic potential of nucleosides as drugs for treating patients with liver diseases. Often these limitations arise from either poor conversion of the nucleoside to the corresponding NTP in the liver or to simultaneous conversion of the nucleoside to the NTP in tissues outside of the liver. HepDirect prodrugs represent a novel strategy for targeting NTP production to the liver. Unlike previous NMP prodrugs, HepDirect prodrugs are capable of bypassing the rate-limiting nucleoside kinase in vivo and generating high liver NTP levels. Both properties result from the remarkable stability of HepDirect prodrugs in plasma and most tissues coupled with their ability to be oxidized by the relatively liver-specific CYP, CYP3A, and converted instantaneously to intermediates and products that are retained inside cells. Higher liver NTP levels are expected to improve efficacy, whereas lower NTP levels outside of the liver diminish the risk of dose-related toxicities.[32] Accordingly, HepDirect prodrugs represent a promising new strategy for improving nucleoside- and phosphonate-based drug therapies targeting liver diseases such as hepatitis B, hepatitis C, and hepatocellular carcinoma.

Materials and Methods

General Methods. All moisture-sensitive reactions were performed under a nitrogen atmosphere using flame-dried glassware and anhydrous solvents purchased from Aldrich. TLC was performed on Analtech Uniplate Silica gel GHLF (250 microns, 10 × 20 cm) plates. Flash chromatography was performed on 230−400 mesh EM Science silica gel 60. Melting points were recorded on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on Varian Gemini or Mercury spectrometers operating respectively at 200 or 300 MHz for proton, 50 or 75 MHz for carbon, and 121 MHz for phosphorus spectra. Analytical HPLC was performed on a 4.6 × 250 mm YMC ODS-AQ 5 μm column eluting with a 0.1% aqueous AcOH/MeOH gradient at a flow rate of 1 mL/min and the detector set at 280 nm. Optical rotations were recorded at 20 °C on a Perkin-Elmer model 341 Polarimeter by Robertson Microlit Laboratories, Inc., Madison, NJ. Mass spectra data were determined on a Perkin-Elmer Sciex API2000 LC-MS system. C, H, and N microanalyses were performed by NuMega Resonance Labs, Inc., San Diego, CA, or by Robertson Microlit Laboratories.

(*S*)-(−)-1-(3-Chlorophenyl)-1,3-propanediol (17*S*). Triethylamine (7.8 mL, 56 mmol) was added to a −20 °C suspension of β-ketoacid **18** (10.94 g, 55.12 mmol) in CH_2Cl_2 (120 mL). The resulting pale yellow solution was treated with a solution of (−)-DIP−Cl (22.1 g, 68.9 mmol) in CH_2Cl_2 (120 mL) at a rate that maintained the internal temperature below −20 °C. The temperature was raised to −10 °C, stirred for a period of 4 h, and quenched with water. The pH of the solution was adjusted to >12 by adding 10% sodium hydroxide. After an ether extraction, the aqueous phase was cooled with ice, treated with concentrated HCl to adjust the pH to <1, and extracted with EtOAc. The organic phase was washed with water and brine and dried ($MgSO_4$). Concentration under reduced pressure gave a pale yellow oil, which was dissolved in ether and treated with pentane until a white precipitate formed. The solid was collected by filtration, rinsed with pentane, and dried at room temperature under reduced pressure to yield (*S*)-(−)-3-(3-chlorophenyl)-3-hydroxypropionic acid (7.68 g, 65%). ^{1}H NMR (200 MHz, DMSO-d_6): 12.2 (br s, 1H), 7.5−7.2 (m, 4H), 5.5 (br s, 1H), 4.9 (m, 1H), 2.5 (m, 2H). The acid (16.12 g, 80.4 mmol) was dissolved in THF (50 mL) by heating for 1 h at reflux. The resulting solution was cooled and cannulated into a suspension of $LiAlH_4$ (12.39 g, 326.5 mmol) in THF (100 mL). The mixture was stirred at room temperature for 2 h, cooled in an ice-bath, and treated with solid NaF (157 g) and then a mixture of water (69 mL) and THF (375 mL). The resulting suspension was stirred at room temperature for 2 h and filtered through a Celite pad. The filtrate was concentrated, and the residue was distilled under reduced pressure to afford **17*S*** (13.23 g, 88%) as a pale yellow oil. bp 142−144 °C (0.2 mmHg). ^{1}H NMR (200 MHz, DMSO-d_6): 7.4−7.2 (m, 4H), 5.28 (d, J = 4.4 Hz, 1H), 4.66 (m, 1H),

(28) (a) Neudecker, T.; Eder, E.; Deininger, C.; Hoffman, C.; Henschler, D. *Mutat. Res.* **1989**, *227*, 131−134. (b) Waegemaekers, T. H. J. M.; Bensink, M. P. M. *Mutat. Res.* **1984**, *137*, 95−102.
(29) Fernandez-Checa, J. C.; Lu, S.; Ookhtens, M.; DeLeve, L.; Runnegar, M.; Yoshida, H.; Saiki, H.; Kannan, R.; Maddatu, T.; Garcia-Ruiz, C.; Kuhlenkamp, J.; Kaplowitz, N. *Hepatic Transport and Bile Secretion Physiology and Pathophysiology*; Raven Press, Ltd.: New York, 1993; pp 363−394.
(30) For a review, see: Jackson, C. H.; MacDonald, N. C.; Cornett, J. W. *Can. Med. Assoc. J.* **1984**, *131*, 25−32.
(31) Milam, K. M.; Byard, J. L. *Toxicol. Appl. Pharmacol.* **1985**, *79*, 342−347.
(32) Erion, M. D.; Colby, T. J.; Reddy, K. R.; MacKenna, D. A.; Boyer, S. H.; Fujitaki, J. M.; Linemeyer, D. L.; Bullough, D. A.; van Poelje, P. D. *Hepatology* **2002**, A551.

4.45 (t, $J = 5.13$ Hz, 1H), 3.46 (m, 2H), 1.7 (m, 2H). $[MH]^+$ calcd for $C_9H_{11}ClO_2$: 187. Found: 187. The enantiomeric excess (ee) was determined to be 96% based on chiral HPLC analysis of the diacetates generated from **17** using methods described in the Supporting Information.

(+)-*cis*-9-{2-[4-(*S*)-(3-Chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-methyleneoxy]eth-1-yl}adenine (19). Dichloridate Coupling Procedure. Oxalyl chloride (2.8 mL, 32 mmol) was added slowly to a slurry of PMEA (2.50 g, 9.16 mmol) and *N*,*N*-diethylformamide (1.0 g, 9.9 mmol) in CH_2Cl_2 (70 mL), and the resulting mixture was refluxed for 3 h. The dichloridate formation was monitored by quenching a small aliquot of the reaction mixture with MeOH and analyzing the resulting dimethyl ester by HPLC (see Supporting Information). The reaction was cooled to room temperature and concentrated in vacuo. The resulting yellow foam was dissolved in CH_2Cl_2 (50 mL), cooled to 0 °C, and treated slowly with pyridine (1.5 mL, 18 mmol). The cold solution was then added slowly to a −78 °C solution of diol **17*S*** (1.70 g, 9.16 mmol) and triethylamine (7.4 mL, 58 mmol) in CH_2Cl_2 (30 mL) at a rate that maintained the internal reaction temperature at <−50 °C. The reaction mixture was warmed to 0 °C, washed with water (80 mL), and the organic layer was dried ($MgSO_4$) and concentrated to provide crude **19** as a 3:1 *cis*:*trans* mixture. The crude product was dissolved in EtOH (30 mL), treated with AcOH (2.3 mL), and heated at reflux. After 2 h, the reaction was cooled to room temperature and concentrated. The resulting dark viscous residue was dissolved in EtOAc (20 mL) and allowed to stand for 2.5 h. The resulting mixture was filtered, and the filtrate was concentrated. The residue was dissolved in EtOAc (40 mL) and stirred for 16 h at room temperature. The solid from the filtration was suspended in EtOAc (20 mL), heated at reflux for 1 h, and then stirred overnight at room temperature. Both suspensions were filtered, and the filtrates were combined, concentrated under reduced pressure, and the residue was purified by chromatography two times (8−10% MeOH in CH_2Cl_2) to yield prodrug **19** as a light yellow foam (1.56 g, 40%, >97% *cis*). $R_f = 0.39$ (10% MeOH in CH_2Cl_2). $[\alpha]_D = +44.9°$ (*c* 0.978, MeOH). ^{1}H NMR (200 MHz, DMSO-d_6): 8.08 (s, 1H), 8.07 (s, 1H), 7.43−7.35 (m, 3H), 7.19 (br s, 1H), 5.57 (br dd, $J = 9.5$, 3.4 Hz, 1H), 4.55−4.26 (m, 3H), 4.26−4.05 (m, 1H), 4.01−3.83 (m, 4H), 2.04−1.75 (m, 2H). $[MH]^+$ calcd for $C_{17}H_{19}ClN_5O_4P$: 424. Found: 424. Anal. Calcd for $C_{17}H_{19}ClN_5O_4P$: C, 48.18; H, 4.52; N, 16.53. Found: C, 48.11; H, 4.46; N, 16.26.

***cis*- and *trans*- 5′-*O*-{2-[4-(3-Chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-methyleneoxy]eth-1-yl}-2′,3′-dideoxy-3′-thiacytidine (25 and 26). Procedure Using P(III) Chemistry.** A solution of **16** (25 g, 0.13 mol) in anhydrous THF (700 mL) and triethylamine (82 mL, 0.59 mol) was added dropwise to diisopropylphosphoramidous dichloride (20.7 mL, 0.147 mmol) in anhydrous THF (350 mL) at −78 °C over 2 h. The reaction mixture was warmed to room temperature overnight, filtered, and the solid was washed with THF. The combined filtrate was evaporated under reduced pressure, and the residue was purified by chromatography (5% EtOAc in hexanes). The compound was further purified by distillation at 117−120 °C/0.1 mm to give **24** as a low melting solid (13.5 g, 32%). $R_f = 0.50$ (5% EtOAc in hexanes). mp 44−46 °C. ^{1}H NMR (200 MHz, DMSO-d_6): 7.5−7.2 (m, 4H), 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). A solution of 3TC (500 mg, 2.18 mmol) and **24** (1.2 g, 3.3 mmol) in DMF (20 mL) was treated with 5-(methylthio)-1*H*-tetrazole (380 mg, 3.27 mmol) and stirred for 30 min at room temperature. The reaction mixture was cooled to −40 °C, treated with a 5−6 M solution of *tert*-butylhydroperoxide in heptane (1 mL, 5 mmol), warmed to room temperature, and stirred overnight. Concentration of the reaction mixture under reduced pressure followed by chromatography (10% MeOH in CH_2Cl_2) gave the *trans*-isomer **26** (350 mg, 35%) as a white foamy solid and the *cis*-isomer **25** as a white solid (450 mg, 45%). (**25**): mp 110−113 °C. $R_f = 0.40$ (10% MeOH in CH_2Cl_2). ^{1}H NMR (200 MHz, DMSO-d_6): 7.66 (s, 1H), 7.62 (s, 1H), 7.50−7.21 (m, 4H), 6.22 (t, $J = 6$ Hz, 1H), 5.78−5.62 (m, 3H), 5.42−5.30 (m, 1H), 4.62−4.20 (m, 4H), 3.45−3.24 (m, 1H), 3.18−2.97 (m, 1H), 2.40−2.05 (m, 2H). $[MH]^+$ calcd for $C_{17}H_{19}ClN_3O_6$ PS: 460. Found: 460. Anal. Calcd for $C_{17}H_{19}ClN_3O_6PS\cdot0.75H_2O$: C, 43.14; H, 4.37; N, 8.88. Found: C, 43.00; H, 3.95; N, 8.70. (**26**): $R_f = 0.45$ (10% MeOH in CH_2Cl_2). ^{1}H NMR (200 MHz, DMSO-d_6): 7.80−7.60 (m, 2H), 7.52−7.16 (m, 4H), 6.32−6.16 (m, 1H), 5.80−5.36 (m, 4H), 4.56−4.20 (m, 4H), 3.46−3.22 (m, 1H), 3.18−2.98 (m, 1H), 2.26−2.00 (m, 2H). Anal. Calcd for $C_{17}H_{19}ClN_3O_6PS\cdot0.5H_2O\cdot0.1CH_2Cl_2$: C, 42.82; H, 4.25; N, 8.74. Found: C, 42.71; H, 3.82; N, 8.54.

(+)-*cis*-5′-*O*-[4-(*S*)-(3-Chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]cytosine-1-*β*-D-arabinofuranoside (33). Procedure Using P(V) Chemistry. A solution of **17*S*** (3.11 g, 16.7 mmol) and TEA (8.64 mL, 62 mmol) in THF (50 mL) was added dropwise to a solution of 4-nitrophenoxyphosphorodichloridate (7.93 g, 31 mmol) in THF (5 mL) at 0 °C. The starting diol was consumed in 2 h, resulting in a 60:40 mixture of **17A** and its *cis*-isomer. Additional TEA (8.6 mL, 62 mmol) and 4-nitrophenol (8.63 g, 62 mmol) were added, and the reaction mixture was stirred overnight. The solvent was evaporated, and the residue was partitioned between EtOAc and water. The organic phase was washed with 0.4 M NaOH, water, brine, and dried ($MgSO_4$). Evaporation of the solvent gave a solid, which was purified by chromatography (60% EtOAc in hexanes) to yield **17A** (4.49 g, 72%, >95% *trans*-isomer based on ^{1}H and ^{31}P NMR). mp 114−115 °C. $R_f = 0.22$ (40% EtOAc in hexanes). $[\alpha]_D = -91.71°$ (*c* 1.01, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): 8.26 (d, $J = 9.7$ Hz, 2H), 7.5−7.2 (m, 6H), 5.56 (d, $J = 11.7$ Hz, 1H), 4.7−4.4 (m, 2H), 2.6−2.2 (m, 1H), 2.2−2.0 (m, 1H). $[MH]^+$ calcd for $C_{15}H_{13}NO_6ClP$: 370. Found: 370. Anal. Calcd for $C_{15}H_{13}NO_6ClP$: C, 48.73; H, 3.54; N, 3.79. Found: C, 48.61; H, 3.36; N, 3.66. A solution of **30** (3.44 g, 7.3 mmol) in DMF (40 mL) was treated with a THF solution of 1 M *tert*-BuMgCl (8.8 mL, 8.8 mmol) and stirred at room temperature. After 30 min, **17A** (4.05 g, 10.9 mmol) was added in one portion. The reaction mixture was stirred for 18 h at room temperature and then quenched with saturated NH_4Cl (20 mL) and extracted with EtOAc. The organic extract was washed with 1 N NaOH, brine, dried (Na_2SO_4), and concentrated to a residue, which was purified by chromatography (5−10% MeOH in CH_2Cl_2) to give diTBDMS protected **33** (2.04 g, 40%). ^{1}H NMR (200 MHz, $CDCl_3$): 7.81 (d, $J = 7.7$ Hz, 1H), 7.4−7.2 (m, 4H), 6.27 (br s, 1H), 5.83 (br s, 1H), 5.64 (d, $J = 8.8$ Hz, 1H), 4.8−4.6 (m, 1H), 4.6−4.3 (m, 2H), 4.3−4.1 (m, 3H), 2.4−2.0 (m, 2H), 0.79 (s, 9H), 0.78 (s, 9H), 0.091 (s, 3H), 0.03 (s, 3H), 0.02 (s, 3H), −0.15 (s, 3H). A solution of the protected prodrug (1.67 g, 2.4 mmol) in THF (30 mL) was treated with TBAF (2.35 g, 3.75 mmol). The resulting heterogeneous mixture was stirred for 18 h at room temperature and then concentrated. The residue was purified by chromatography (5−15% MeOH in CH_2Cl_2) to yield **33** as a white solid (440 mg, 39%). mp >200 °C. $[\alpha]_D = +55.16°$ (*c* 0.925, MeOH). ^{1}H NMR (200 MHz, DMSO-d_6): 7.52 (d, $J = 7.32$ Hz, 1H), 7.5−7.2 (m, 4H), 7.07 (br s, 2H), 6.10 (m, 1H), 5.8−5.5 (m, 4H), 4.6−4.2 (m, 4H), 4.0−3.8 (m, 3H), 2.3−2.1 (m, 2H). $[MH]^+$ calcd for $C_{18}H_{21}N_3O_8ClP$: 474. Found: 474. Anal. Calcd for $C_{18}H_{21}N_3O_8ClP\cdot0.8H_2O$: C, 44.28; H, 4.67; N, 8.61. Found: C, 44.32; H, 4.47; N, 8.42.

Biological Procedures

Materials. Pooled male rat (Sprague-Dawley) microsomes and recombinant CYP Supersomes were purchased from Discovery Labware, Inc. (Woburn, MA). Mixed human liver microsomes, prepared from a pool of 15 male and female donors, were purchased from In Vitro Technologies, Inc. (Baltimore, MD). AraA, araAMP, and araATP were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). 3TC was purchased from Sav-Mart pharmacy and purified by silica gel chromatography at Metabasis Therapeutics, Inc. PMEA-DP and araCTP were purchased from Trilink Biotechnology, Inc. (San Diego, CA). 3TC-TP was purchased from Moravek, Inc. (Brea, CA).

Byproduct Capture Assay. Reaction mixtures consisting of 100 mM KH_2PO_4, 10 mM glutathione, microsomal protein (0.5−2.5 mg/

mL rat or human liver microsomes or 100 pmol/mL recombinant human microsomes) were preincubated for 2 min in an Eppendorf Thermomixer (600 rpm, 37 °C). Reactions were initiated by addition of 1 mM NADH and quenched by adding 1.5 volumes of methanol. After centrifugation (14 000 rpm, 10 min), the supernatants were incubated at 50 °C for 4 h. Samples were loaded onto a Beckman Ultrasphere 5μ C_{18} column (4.6 × 250 mm) in 90% 20 mM potassium phosphate buffer (pH 6.2) and 10% acetonitrile and then eluted with a gradient of 10−60% acetonitrile over 20 min. The conjugate of glutathione and 3-Cl-phenylvinyl ketone (i.e., **38**) eluted with a retention time of 10 min. The acrolein byproduct capture assay for IFA was conducted as previously described.[33]

Hepatocyte Isolation and Assays. Rat hepatocytes were isolated from Sprague-Dawley rats using a modified literature procedure.[34] Compounds were incubated in suspended hepatocytes for 4 h at 37 °C and then extracted by brief centrifugation (10 000 rpm in microfuge) through a mineral/silicon (4:1) oil layer into 10% perchloric acid. The perchloric acid layer was centrifuged for 10 min (14 000 rpm). The resulting supernatant was neutralized with 0.3 volumes of 3 M KOH/3 M $KHCO_3$ and analyzed by ion exchange HPLC as described below.

In Vivo Studies. Dosing solutions of 3TC and **25** were prepared in isotonic saline at a drug concentration of 230 mg/mL and in 75% PEG-400/saline at a drug concentration of 30 mg/mL (3TC equivalents), respectively. Groups of four rats were administered either 30 mg/kg of 3TC equivalents (1 mL/kg) of **25** or 230 mg/kg of 3TC (3 mL/kg) by iv bolus via the tail vein under light halothane anesthesia. At prespecified times, rats were anesthetized and 1 mL of heparinized blood was obtained from the abdominal vena cava and centrifuged (14 000 rpm on tabletop microfuge) to isolate plasma. Liver samples were freeze-clamped and homogenized with 3 volumes (per weight) of ice-cold 10% perchloric acid. After centrifugation at 2600*g* for 10 min (3000 rpm on Sorvall RC-3B+) at 5 °C, aliquots (1 mL) of the supernatants were neutralized with 0.3 mL of ice-cold 3 M potassium carbonate and stored frozen until analysis by HPLC. The neutralized acid extracts were analyzed for 3TC-TP as described below.

NTP Analysis. Neutralized hepatocyte extracts were analyzed for NTPs on a Whatman Partisil SAX column (4.6 × 250 mm) eluted with a linear gradient of Buffer A (10 mM ammonium phosphate pH 3.5 and 6% v/v ethanol) and Buffer B (1 M ammonium phosphate pH 3.5 and 6% v/v ethanol) [30−80% Buffer B over 0−25 min] at a flow rate of 1.25 mL/min. Extracts containing araCTP were treated with sodium periodate to remove interfering ribonucleoside triphosphates. NTP elution was monitored by UV absorbance at 254 nm for araATP (13 min) and PMEA-DP (19 min) and 270 nm for 3TC-TP (16 min) and araCTP (12 min).

Prodrug Cleavage Intermediate Identification. Rat liver microsomes and isolated rat hepatocytes were incubated with **21** and **25** for 5 min and 2 h, respectively, and extracted with methanol. Analysis of the extracts by HPLC using the method described above showed a new peak at 9.5 min in samples from incubations performed with **21**. A similar peak was observed during analysis of urine collected from ICR mice treated with **21** (1000 mg/kg, ip). The mass for the metabolite was determined after HPLC purification as follows. Urine was deproteinated by addition of two volumes of methanol (100%), vortexing and centrifugation (14 000 rpm, room temperature, 5 min). One hundred microliters of the supernatant was injected onto a C_{18} RP HPLC column equilibrated with 90% ammonium formate buffer (10 mM, pH 6.3) and 10% acetonitrile. The column was then eluted with an acetonitrile gradient (10−25% over 15 min). The fraction corresponding to an elution time of 8−8.5 min was collected and evaporated to dryness in a Savant speed vacuum/cold trap and then resuspended in methanol. The sample was analyzed by Mass Consortium Corp. (San Diego, CA) by electrospray MS and shown to have a MW of 441. Stability studies for the purified metabolite were conducted in 100 mM ammonium formate pH 6.3 and in 100 mM sodium phosphate pH 9.0.

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Supporting Information Available: Analytical data and synthetic procedures for PMEA prodrugs **20**−**23**, araA prodrugs **31**−**32**, and araC prodrugs **34**−**36**, and their corresponding precursors, including 1,3-diols **16** and **17** (*R*-isomer), P(V) phosphate precursors **27A**−**28A**, and protected nucleosides **29**−**30**. Procedure for determining ee for diols. Analytical data and synthetic procedures for prodrug cleavage intermediate **37**, aryl vinyl ketone glutathione conjugate **38**, and ring-opened degradants **39**−**40**. Procedures for liver microsome studies, CYP inhibition studies, and recombinant CYP enzyme kinetics. Procedures for prodrug aqueous plasma and tissue stability and aqueous solubility determinations. Assays for 3TC, PMEA, and araAMP and metabolites (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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