Liver-Targeted Prodrugs of 2′-*C*-Methyladenosine for Therapy of Hepatitis C Virus Infection

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2′-*C*-Methyladenosine exhibits impressive inhibitory activity in the cell-based hepatitis C virus (HCV) subgenomic replicon assay, by virtue of intracellular conversion to the corresponding nucleoside triphosphate (NTP) and inhibition of NS5B RNA-dependent RNA polymerase (RdRp). However, rapid degradation by adenosine deaminase (ADA) limits its overall therapeutic potential. To reduce ADA-mediated deamination, we prepared cyclic 1-aryl-1,3-propanyl prodrugs of the corresponding nucleoside monophosphate (NMP), anticipating cytochrome P450 3A-mediated oxidative cleavage to the NMP in hepatocytes. Lead compounds identified in a primary rat hepatocyte screen were shown to result in liver levels of NTP predictive of efficacy after intravenous dosing to rats. The oral bioavailability of the initial lead was below 5%; therefore, additional analogues were synthesized and screened for liver NTP levels after oral administration to rats. Addition of a 2′,3′-carbonate prodrug moiety proved to be a successful strategy, and the 1-(4-pyridyl)-1,3-propanyl prodrug containing a 2′,3′-carbonate moiety displayed oral bioavailability of 39%.

Introduction

Hepatitis C virus (HCV)[a] infection is a serious worldwide health problem that according to the World Health Organization affects 2% of the world population.[1] While the early stages of the disease are typically asymptomatic, the majority of HCV infections progress to chronic infection, which is associated with an increased risk of cirrhosis, hepatocellular carcinoma, and liver failure. At present, the only therapy available for patients with chronic HCV infection is a combination of ribavirin and interferon-based therapies, which leads to a sustained virologic response in only about half the patients treated.[2]

Efforts to discover more effective drugs to treat HCV-infected patients have focused on several possible targets, including the NS5B RNA-dependent RNA polymerase (RdRp).[3,4] A number of nucleoside analogues are reported to inhibit RdRp (as the corresponding triphosphate) in enzyme and cell-based assays, and particularly nucleoside analogues containing the 2′-*C*-methylribose sugar.[5] Within this group, 2′-*C*-methyladenosine (**1**, Figure 1) has shown impressive activity in in vitro systems such as the cell-based HCV subgenomic replicon assay ($EC_{50} = 0.3\ \mu M$).[6] However, pharmacokinetic studies have shown very rapid clearance of **1** upon intravenous dosing in rats, and further studies to investigate the reason revealed that **1** is an efficient substrate for adenosine deaminase (ADA).[6−8]

The challenge of circumventing metabolic degradation and achieving delivery of the intact nucleoside to the liver is particularly well-suited to a prodrug approach previously reported from these laboratories, which delivers the monophosphate form (NMP) specifically to cells expressing CYP 3A such as hepatocytes (Figure 2).[9,10] Oxidation of the prodrug moiety by CYP 3A yields a hemiketal, which spontaneously isomerizes to a ring-opened form and undergoes β-elimination to yield the NMP. This delivery approach bypasses the initial nucleoside phosphorylation step known to be rate-limiting in the conversion of many nucleosides to their active NTP form. By selectively enhancing NTP generation in the liver, prodrugs of this type may offer an improved therapeutic index, particularly if the metabolic fate of the NTP involves a path other than conversion back to nucleoside and release from the liver into the bloodstream.

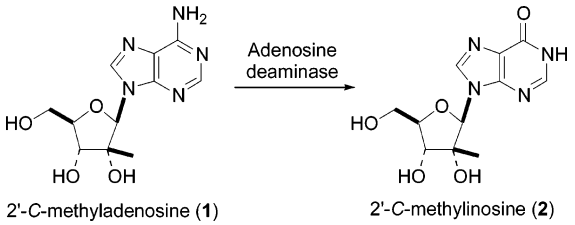

Figure 1. Conversion of 2′-*C*-methyladenosine (**1**) to 2′-*C*-methylinosine (**2**) by ADA.

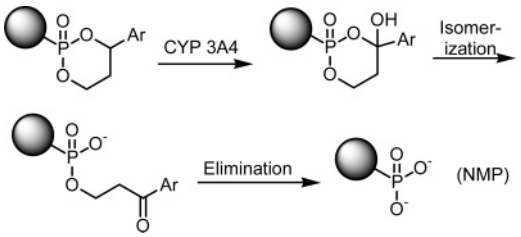

Figure 2. Mechanism of activation of cyclic 1-aryl-1,3-propanyl (HepDirect) prodrugs.

It has been reported that ADA requires a free 5′-OH group for processing of substrates,[11] so it would be expected that 1-aryl-1,3-propanyl 5′-monophosphate prodrugs would avoid metabolic degradation by this enzyme. Armed with this

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[a] Abbreviations: HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; ADA, adenosine deaminase; NTP, nucleoside triphosphate; NMP, nucleoside monophosphate; DMSO, dimethyl sulfoxide; LC-MS/MS, liquid chromatography with tandem mass spectrometry.

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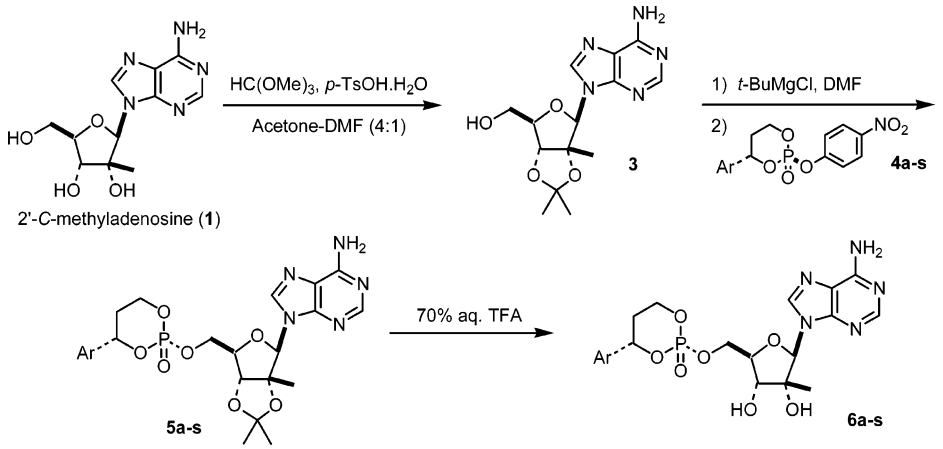


Figure 3. Synthesis of 1-aryl-1,3-propanyl monophosphate prodrugs of **1**.

rationale, a program was initiated whose objective was to discover a prodrug of 2′-*C*-methyladenosine that would achieve therapeutic concentrations of nucleoside triphosphate (NTP) in the liver upon oral administration.

Results and Discussion

Synthesis of Prodrugs. The synthesis of prodrugs of 2′-*C*-methyladenosine was carried out as depicted in Figure 3. The nucleoside was protected as its 2′,3′-isopropylidene ketal **3** and was then phosphorylated at 5′ with *trans-p*-nitrophenylphosphate reagents **4a**−**s**.[9,12] Removal of the protecting group from compounds **5a**−**s** afforded the desired *cis*-prodrugs **6a**−**s**. Yields (unoptimized) for the two-step conversion of **3** to **6a**−**s** were generally 25−60%, although in a few cases lower yields were obtained. Previous work had shown that an electron-withdrawing group on the phenyl ring is important for sufficient chemical stability, so only analogues of this type were prepared.[9] In most cases, the phosphorylating reagents **4a**−**s** were prepared in racemic form, and therefore the prodrugs **6a**−**s** are a mixture of two *cis*-diastereomers; a single *cis*-diastereomer was prepared in the cases of 3-chlorophenyl prodrug **6a** and 4-pyridyl prodrug **6p**, since the corresponding phosphorylation reagents **4a** and **4p** were available as single enantiomers from other projects.[10,13] Yield and purity data, as well as results of mass spectrometric (MS) analysis confirming the structures, are shown in Table 1.

Activation in Rat Hepatocytes. Activation of prodrugs **6a**−**s** to the corresponding NTPs, which requires prodrug cleavage as well as two phosphorylation events catalyzed by cellular nucleotide kinases, was evaluated in rat hepatocytes (Table 2). Many prodrugs were efficiently cleaved and phosphorylated, affording levels of NTP comparable to those derived from the unmodified nucleoside **1**. The highest activation levels were seen with halo-substituted phenyl prodrugs, with the highest level observed with dichlorophenyl prodrug **6c**. Pyridyl prodrugs **6n**−**s** were also activated but gave lower levels of NTP. The beneficial effect of halogen substitution was noted in the pyridyl series as well, as seen with 5-bromo-3-pyridyl derivative **6q**.

Evaluation of Liver NTP Levels in Rat. On the basis of the intracellular concentration of NTP in the replicon assay at the EC_{90}, we sought to achieve a concentration of NTP in the liver of 30 nmol/g.[14] Levels of NTP generated in the liver following administration of **1** and its prodrug **6a** were evaluated in the rat (Figure 4); doses of prodrugs were normalized to nucleoside equivalents (ne), that is, the quantity of nucleoside contained. Oral dosing of **1** resulted in negligible levels of the corresponding NTP in liver, whereas intravenous dosing afforded modest exposure at the level of 10 nmol/g. Importantly, intravenous administration of prodrug **6a** achieved liver levels of NTP of up to 50 nmol/g, with sustained exposure above our target of 30 nmol/g for 12 h.

Table 1. Yield and Purity Data for Compounds **6a**−**s**, **9a**−**c**, **9f**, **9g**, **9i**, and **9p**

compd	yield (%) **3** → **6**	yield (%) **6** → **9**	HPLC area (%)	MS
6a	35		94.9	512.0
6b	25		95.4	546.0
6c	30		93.8	546.0
6d	8		96.0	558.0
6e	8		96.8	556.0
6f	20		93.4	496.0
6g	61		93.4	514.2
6h	39		95.1	514.0
6i	25		93.9	514.0
6j	41		93.3	530.0
6k	30		94.0	573.8
6l	35		94.8	503.1
6m	66		93.8	503.0
6n	7		90.1	479.0
6o	30		99.5	479.0
6p	48		94.4	479.0
6q	40		97.4	557.0
6r	12		95.4	509.1
6s	40		94.6	493.1
9a		72	96.0	538.0
9b		76	95.0	572.0
9c		45	94.3	572.0
9f		45	97.8	522.0
9g		95	92.2	540.2
9i		80	98.0	540.0
9p		79	95.6	505.4

Next, prodrug **6a** was evaluated orally at a dose 5-fold higher than used intravenously (iv). Liver levels of NTP were below 10 nmol/g; the oral bioavailability of **6a** based on the AUC of NTP in liver following oral versus intravenous dosing was less than 5%.

In response to the observation of low oral bioavailability, selected prodrugs were screened in the rat for liver levels of NTP at the 3 h time point following an oral dose of 50 mg/kg. None of the prodrugs came close to achieving target levels of NTP in liver, as shown in Table 4. The low liver NTP levels for these compounds are likely due to limited oral absorption, resulting from their high molecular weight and multitude of polar functional groups.

Optimization of Oral Bioavailability. Encouraged by the results of iv dosing of **6a**, we embarked on a program to further functionalize this compound in order to achieve better oral absorption. One approach used to improve the oral bioavailability of nucleoside drugs is attachment of an amino acid

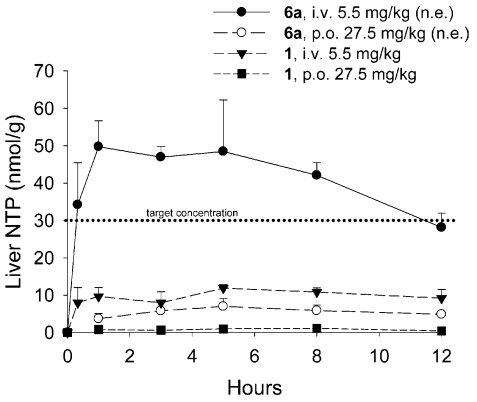


Figure 4. Rat liver levels of 2-*C*-methyladenosine triphosphate after dosing with nucleoside **1** and prodrug **6a**.

Table 2. Activation of Prodrugs **6a**−**s** in Rat Hepatocytes (25 μM, 2 h)

compd	Ar	[NTP], nmol/g
1		209
6a	(*S*)-3-Cl-phenyl	389
6b	3,4-Cl_2-phenyl	396
6c	3,5-Cl_2-phenyl	585
6d	2-Br-phenyl	122
6e	3-Br-phenyl	99
6f	3-F-phenyl	328
6g	2,3-F_2-phenyl	323
6h	2,5-F_2-phenyl	139
6i	3,5-F_2-phenyl	250
6j	4-Cl-3-F-phenyl	328
6k	2-Br-5-F-phenyl	72
6l	3-CN-phenyl	137
6m	4-CN-phenyl	41
6n	2-pyridyl	93
6o	3-pyridyl	73
6p	(*S*)-4-pyridyl	47
6q	5-Br-3-pyridyl	194
6r	2-OMe-4-pyridyl	93
6s	5-Me-3-pyridyl	73

Table 3. Activation of Prodrugs **9a**−**c**, **9f**, **9g**, **9i**, and **9p** in Rat Hepatocytes (25 μM, 2 h)

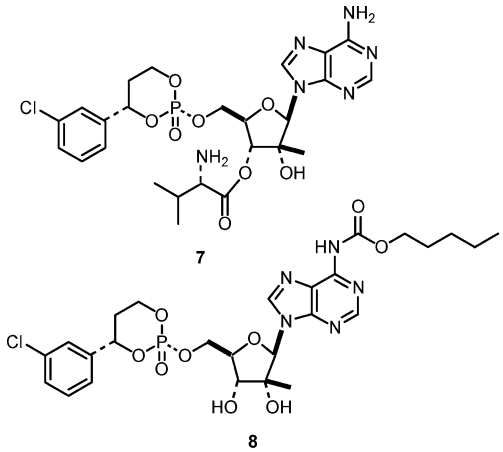
compd	Ar	[NTP], nmol/g
9a	(*S*)-3-Cl-phenyl	296
9b	3,4-Cl_2-phenyl	97
9c	3,5-Cl_2-phenyl	49
9f	3-F-phenyl	313
9g	2,3-F_2-phenyl	280
9i	3,5-F_2-phenyl	113
9p	(*S*)-4-pyridyl	185

Table 4. Rat Liver NTP Levels at 3 h Following Oral Dosing of Prodrugs at 50 mg/kg

	HepDirect alone		HepDirect 2,3-carbonate	
Ar	compd	[NTP], nmol/g	compd	[NTP], nmol/g
(*S*)-3-Cl-phenyl	**6a**	2.9	**9a**	29.2
3,4-Cl_2-phenyl	**6b**	2.0	**9b**	11.8
3,5-Cl_2-phenyl	**6c**	2.4	**9c**	5.0
3-F-phenyl	**6f**	4.1	**9f**	22.9
2,3-F_2-phenyl	**6g**	8.2	**9g**	14.7
3,5-F_2-phenyl	**6i**	4.1	**9i**	11.2
(*S*)-4-pyridyl	**6p**	3.6	**9p**	28.3

residue, such as valine, in order to facilitate uptake by amino acid transporters.[15] Thus, the 3′-valine ester **7** (Figure 5) was prepared; it afforded reasonable activation to NTP in hepatocytes but did not lead to an increase in liver NTP levels (relative to **6a**) following oral administration.

A second approach for improving oral absorption surrounded reducing the number of hydrogen-bond donors by appending



	6a	**7**	**8**
Rat hepatocyte activation[a] (nmol/g)	389	117	73
Liver NTP level, p.o.[b] (nmol/g)	2.9	3.1	1.6

[a] 25 μM, 2 h
[b] 50 mg/kg, 3 h

Figure 5. Initial prodrugs to improve oral bioavailability.

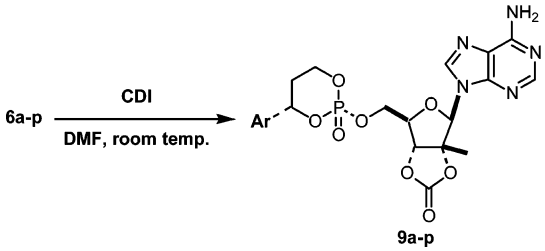


Figure 6. Synthesis of carbonate prodrugs.

acyl groups to the amine and hydroxy functionalities of the nucleoside. Carbamate derivatives of the amino group of cytosine-containing nucleosides have been shown to be efficiently cleaved in vivo.[16] To this end, *n*-pentyl carbamate **8** (Figure 5) was synthesized; like valine ester **7**, it showed good conversion to the NTP in rat hepatocytes but failed to offer a corresponding increase in liver levels upon oral administration to rats.

As we initiated efforts to modify the hydroxy groups of the sugar moiety, we conceived the use of a 2′,3′-carbonate prodrug, which would remove two H-bond donors with only a minor increase in molecular weight. We were gratified to find that 2′,3′-carbonate **9a** afforded a 10-fold increase in rat liver NTP levels upon oral dosing, and efforts were initiated to explore the generality of application of this functional group to 1-aryl-1,3-propanyl monophosphate prodrugs of **1**.

2′,3′-Carbonate Derivatives of 6a−**p.** The 2′,3′-carbonate derivatives **9a**−**p** were prepared by treatment of prodrugs **6a**−**p** with carbonyl diimidazole in *N*,*N*-dimethylformamide (DMF) (Figure 6); yield and purity data are provided in Table 1. The activation of these prodrugs to the NTP in rat hepatocytes is shown in Table 3. In general, the addition of the 2′,3′-carbonate group does not hamper formation of the NTP. However, reduction in activation was observed with 3,5-dichlorophenyl prodrugs **9b** and **9c**, while improvement in the level of activation was seen with 4-pyridyl prodrug **9p**.

Application of the 2′,3′-carbonate in all cases resulted in a marked improvement in oral bioavailability, although the degree

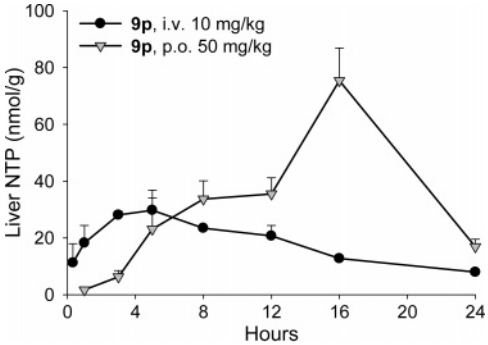


Figure 7. Liver levels of 2′-*C*-Me-ATP after dosing of rats with prodrug **9p**.

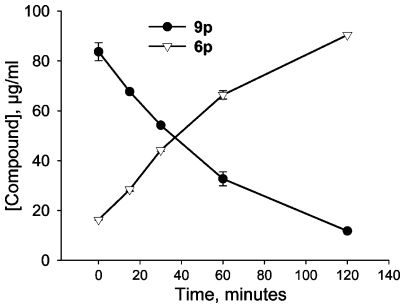


Figure 8. Stability of compound **9p** in rat plasma at 37 °C.

of enhancement varied (Table 4). The highest liver levels of NTP were observed with 3-chlorophenyl prodrug **9a** and 4-pyridyl prodrug **9p**. Further profiling of these two compounds showed that **9p** had 3-fold higher aqueous solubility (0.45 vs 0.16 mg/mL in pH 5.1 citrate buffer), and therefore **9p** was selected for a more extensive pharmacokinetic evaluation.

Liver levels of NTP following intravenous (10 mg/kg) and oral (50 mg/kg) dosing of dual prodrug **9p** in rats are shown in Figure 7. In this experiment, absorption appears to have been delayed relative to the observations in the earlier screening assay, as the NTP level at the 3 h time point is only ∼5 nmol/g versus 28.3 nmol/g in the screening assay. This difference cannot be attributed to formulation, since the same vehicle (PEG-400) was used in both experiments. The maximum liver NTP concentration was achieved at a surprisingly late time point of 16 h,[17] an observation that may be attributable in part to the fact that food was returned to the fasted rats a few hours after dosing. Most importantly, the target concentration of 30 nmol/g is achieved during the period of 8−16 h. The oral bioavailability of **9p**, calculated by comparing AUCs of NTP in liver following oral and intravenous dosing, is 39%.

Compound **9p** shows adequate chemical stability, with a half-life of 13 h in pH 7 buffer at 25 °C. In rat plasma at 37 °C, **9p** is efficiently converted to des-carbonate **6p** (Figure 8), indicating that cleavage is primarily enzyme-mediated. Compound **6p** undergoes no further degradation under these conditions. Although we have not performed studies to identify the enzyme responsible for hydrolysis of the 2′,3′-carbonate moiety, the enzyme paraoxonase has been implicated in the hydrolysis of cyclic carbonates.[18,19]

Conclusions

Application of the prodrug approach described herein successfully circumvents the rapid metabolism of 2′-*C*-methyladenosine, achieving target concentrations of the triphosphate in the liver following intravenous dosing. The use of an additional prodrug, the 2′,3′-carbonate, affords a dramatic increase in oral bioavailability. Dual prodrug **9p** displays properties warranting its further study as a potential new agent for treating HCV.

Experimental Section

General Information. Glassware for moisture-sensitive reactions was flame-dried and cooled to room temperature in a desiccator, and all reactions were carried out under an atmosphere of nitrogen. Anhydrous solvents were purchased from Aldrich or Acros. Thin-layer chromatography was performed on EM Science silica gel 60 F_{254} plates and visualized with a UV lamp (254 nm). Column chromatography was performed on 230−400 mesh EM Science silica gel 60. ^{1}H and ^{13}C NMR were obtained on a Varian Mercury-300 operating at 300 and 75 MHz, respectively. ^{1}H and ^{13}C NMR spectra were recorded in δ units with the solvent's chemical shift as the reference line. C, H, N microanalyses were performed by Robertson Microlit Laboratories, Inc., Madison, NJ, or NuMega Resonance Labs, Inc. San Diego, CA. Purity was assessed on a Hitachi HPLC instrument (L-6000 pump, L-4000 UV detector) with a YMC-ODS-AQ column (250 × 4.6 mm); gradient elution was employed, with 0.05% trifluoroacetic acid/water and methanol at a flow rate of 1.0 mL/min (detector wavelength 280 nm). Mass spectral data were acquired on a PE Sciex API-2000 LC/MS instrument, operating in the positive ion mode. Centrifugations were performed in an Eppendorf microcentrifuge at 14 000 rpm in a cold room. All protocols involving animal experimentation were reviewed and approved by the Metabasis Therapeutics IACUC (Institution Animal Care and Use Committee) and follow the guidelines established by the NRC Guide for the Care and Use of Laboratory Animals.

***cis*-5-*O*-[4-(*S*)-(3-Chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-2′-*C*-methyladenosine, Trifluoroacetic Acid Salt (6a): (A) Phosphorylation.** To a solution of 2′-*C*-methyladenosine-2′,3′-acetonide[20] (3, 1.04 g, 3.24 mmol) in DMF (20 mL), being stirred under nitrogen at 0 °C, was added a 1 M solution of *t*-butylmagnesium chloride in tetrahydrofuran (THF) (5.50 mL, 5.50 mmol). After 20 min, *trans*-4-[(*S*)-3-chlorophenyl]-2-(4-nitrophenoxy)-2-oxo-1,3,2-dioxaphosphorinane[9] (4a, 1.41 g, 3.87 mmol) was added. The mixture was allowed to warm to room temperature and was stirred for 3 h. The solvent was removed with a rotary evaporator, and the residue was subjected to column chromatography on silica gel (5−8% methanol in dichloromethane), affording 1.00 g (1.82 mmol, 56%) of compound **5a**.

(B) Deprotection. Compound **5a** (1.00 g, 1.82 mmol) was added to 70% aqueous trifluoroacetic acid at 0 °C. After being stirred overnight at 0 °C, the mixture was allowed to warm to room temperature and was stirred for 4 h. The volatiles were removed with a rotary evaporator, and the residue was partitioned between 20% methanol in ethyl acetate and saturated aqueous sodium bicarbonate solution. The aqueous layer was extracted three times with 20% methanol in ethyl acetate, and the combined organic phase was washed with brine, dried over sodium sulfate, and filtered; the solvent was removed with a rotary evaporator. The crude product was subjected to column chromatography on silica gel (5−10% methanol in dichloromethane), affording 703 mg (62%) of compound **6a** as an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.24 (s, 1H), 8.16 (s, 1H), 7.45 (s, 2H), 7.3−7.4 (m, 4H), 6.01 (s, 1H), 5.73 (m, 1H), 5.55 (d, J = 10 Hz, 1H), 5.40 (s, 1H), 4.4−4.6 (m, 4H), 4.1−4.25 (m, 2H), 2.19 (m, 2H), 0.82 (s, 3H). Anal. (following repurification to remove residual TFA; $C_{20}H_{23}ClN_5O_7P$) C, H, N.

***cis*-5′-*O*-[4-(*S*)-(Pyridin-4-yl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-2′-*C*-methyladenosine (6p): (A) Phosphorylation.** To a solution of 2′-*C*-methyladenosine-2′,3′-acetonide (**3**, 171 mg, 0.530 mmol) in DMF (5 mL), being stirred under nitrogen, was added a 1 M solution of *t*-butylmagnesium chloride in THF (1.00 mL, 1.00 mmol). After 30 min, a solution of *trans*-4-[(*S*)-pyridin-4-yl]-2-(4-nitrophenoxy)-2-oxo-1,3,2-dioxaphosphorinane[9] (**4p**, 220 mg, 0.65 mmol) was added in one portion. After being stirred at room temperature overnight, the mixture was concentrated with a rotary evaporator, and the residue was subjected to column chromatog-

raphy on silica gel (5−10% methanol in dichloromethane), affording 168 mg (0.323 mmol, 61%) of compound **5p**.

(B) Deprotection. Compound **5p** (168 mg, 0.323 mmol) was added to 70% aqueous trifluoroacetic acid at 0 °C. After being stirred overnight at 0 °C, the mixture was concentrated with a rotary evaporator, and the residue was subjected twice to column chromatography on silica gel (5−12.5% methanol in dichloromethane containing 1% ammonium hydroxide, then 8−10% methanol in acetonitrile containing 1% ammonium hydroxide), affording 120 mg (78%) of compound **6p** as an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.55 (d, J = 4 Hz, 2H), 8.25 (s, 1H), 8.20 (s, 1H), 7.36 (m, 4H), 6.02 (s, 1H), 5.74 (m, 1H), 5.51 (d, J = 10 Hz, 1H), 5.40 (s, 1H), 4.4−4.6 (m, 4H), 4.1−4.3 (m, 2H), 2.0−2.3 (m, 2H), 0.83 (s, 3H). Anal. ($C_{19}H_{23}N_6O_7P\cdot 2.5H_2O$) C, H, N.

***cis*-5′-O-[4-(*S*)-(3-Chlorophenyl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-2′-*C*-*β*-methyladenosine-2′,3′-carbonate (9a).** To a solution of compound **6a** (0.80 g, 1.56 mmol) in THF (15 mL), being stirred at room temperature, was added 1,1′-carbonyldiimidazole (0.70 g, 4.32 mmol). After 3 h, the solvent was removed with a rotary evaporator, and the residue was subjected to column chromatography on silica gel (5−6% methanol in dichloromethane), affording 0.60 g (72%) of compound **9a** as an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.20 (s, 1H), 8.16 (s, 1H), 7.3−7.5 (m, 6H), 6.59 (s, 1H), 5.72 (m, 1H), 5.08, (d, J = 4 Hz, 1H), 4.35−4.65 (m, 5H), 2.1−2.3 (m, 2H), 1.25 (s, 3H). Anal. ($C_{21}H_{21}N_5O_8ClP\cdot 1.0H_2O$) C, H, N.

***cis*-5′-*O*-[4-(*S*)-(Pyridin-4-yl)-2-oxo-1,3,2-dioxaphosphorinan-2-yl]-2′-*C*-methyladenosine-2′,3′-carbonate (9p).** To a solution of compound **6p** (600 mg, 1.25 mmol) in DMF (10 mL), being stirred at 0 °C, was added 1,1′-carbonyldiimidazole (407 mg, 2.51 mmol). The mixture was allowed to warm to room temperature and was stirred for 3 h. The solvent was removed with a rotary evaporator, and the residue was subjected to column chromatography on silica gel (0−10% methanol in dichloromethane), affording 513 mg (79%) of compound **9p** as an off-white solid. ^{1}H NMR (DMSO-d_6) δ 8.57 (d, J = 4 Hz, 2H), 8.22 (s, 1H), 8.16 (s, 1H), 7.44 (s, 2H), 7.37 (d, J = 4 Hz, 2H), 6.59 (s, 1H), 5.76 (m, 1H), 5.08 (d, J = 4 Hz, 1H), 4.35−4.65 (m, 5H), 2.1−2.3 (m, 2H), 1.25 (s, 3H). Anal. ($C_{20}H_{21}N_6O_8P\cdot 1.2H_2O$) C, H, N.

NTP Generation in Rat Hepatocytes. Hepatocytes were prepared from fed Sprague−Dawley rats (250−300 g) according to the procedure of Berry and Friend[21] as modified by Groen et al.[22] Hepatocytes (20 mg/mL wet weight, >85% trypan blue viability) were incubated at 37 °C in 2 mL of Krebs−bicarbonate buffer containing 20 mM glucose and 1 mg/mL bovine serum albumin (BSA) for 2 h in the presence of 1−250 μM nucleoside or prodrug (from 25 mM stock solutions in DMSO). Following the incubation, a 1600 μL aliquot of the cell suspension was centrifuged and 300 μL of acetonitrile was added to the pellet, which was vortexed and sonicated until the pellet broke down. Then 200 μL of water was added to make a 60% acetonitrile solution. After 10 min of centrifugation at 14 000 rpm, the resulting supernatant was transferred to a new vial and evaporated to near dryness in a Savant SpeedVac Plus at room temperature. The dried residue was reconstituted with 200 μL of water and the mixture was centrifuged for 10 min at 14 000 rpm. A mixture of 35 μL of supernatant and 35 μL of mobile phase A (20 mM *N*,*N*-dimethylhexylamine and 10 mM propionic acid in 20% methanol) was analyzed by LC-MS/MS (Applied Biosystems, API 4000) equipped with an Agilent 1100 binary pump and a LEAP injector. NTP was detected by using MS/MS mode (M^-/78.8) and quantified on the basis of comparison to a standard of 2′-*C*-methyladenosine-5′-triphosphate.

Evaluation of Liver NTP Levels in Rat. Nucleoside analogues and their prodrugs were administered to Sprague−Dawley rats by oral gavage. At 2 or 3 h following drug administration, liver samples (∼1 g) were collected, snap-frozen, and homogenized in 3 volumes of ice-cold 70% methanol containing 20 mM EDTA/EGTA. Following centrifugation to clarify the homogenate, aliquots of the supernatants (100 μL) were evaporated to dryness on a Savant Speed-Vac Plus (1 h, room temperature). The resulting dried residue was reconstituted with 100 μL of mobile phase and then analyzed for nucleotides by an LC-MS/MS method as described below.

The reconstituted extracts in mobile phase A (20 mM *N*,*N*-dimethylhexylamine and 10 mM propionic acid in 20% methanol) were analyzed by LC-MS/MS (Applied Biosystems, API 4000) equipped with an Agilent 1100 binary pump and a LEAP injector. Ten microliters of sample was injected onto an Xterra MS C18 column (3.5 um, 2.1 × 50 mm, Waters Corp.) with a SecurityGuard C18 guard column (5 μm, 4.0 × 3.0 mm, Phenomenex) and eluted with a gradient of mobile phases A and B (20 mM *N*,*N*-dimethylhexylamine and 10 mM propionic acid in 80% methanol) at a flow rate of 0.3 mL/min (0 min, 0% B, 0−1 min, 0−50% B; 1−3 min, 50−100% B, 3−6 min, 100% B; 6−6.1 min, 100−0% B; 6.1−9 min, 0% B). NTP was detected by using MS/MS mode (M^-/78.8). The quantitative analysis of liver NTP was calculated on the basis of a calibration curve generated with an authentic standard of 2′-*C*-methyladenosine triphosphate (0.01, 0.03, 0.1, 0.3, 1.0, 3, 10, and 30 μM).

Evaluation of Stability in Blood and Plasma. 2′-*C*-Methyladenosine and prodrugs thereof were separately incubated in heparinized whole rat blood or plasma at 37 °C. Aliquots of the blood and plasma samples were removed periodically and extracted with perchloric acid and 0.2% formic acid in acetonitrile, respectively, and then centrifuged (22000*g*, 20 min, 4 °C). The acidic supernatants were neutralized with potassium carbonate and centrifuged again (22000*g*, 20 min, 4 °C). The neutralized supernatants and acetonitrile extracts were then analyzed for the major metabolite of 2′-*C*-methyladenosine, that is, 2′-*C*-methylinosine, as described below.

The plasma extracts were analyzed by HPLC on an Agilent 1100 instrument. Analysis (50 μL) was performed on an Agilent Zorbax SB-Aq column (4.6 × 150 mm) eluted with a gradient consisting of a mixture of buffer A (20 mM potassium phosphate, pH 6.2) and buffer B (acetonitrile) (0−10 min, 0−10% buffer B; 10−20 min, 10−80%, 20−21 min, 80−0%; 21−30 min, 0%) and UV absorbance monitoring at 265 nm. The flow rate was 1.5 mL/min and the column temperature was set at 40 °C. Concentrations of metabolite were determined from calibration curves prepared by spiking known amounts of standards to plasma and processing as before. The LOQ of 2′-*C*-methylinosine was 1 μM.

Supporting Information Available: Results from elemental analysis and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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