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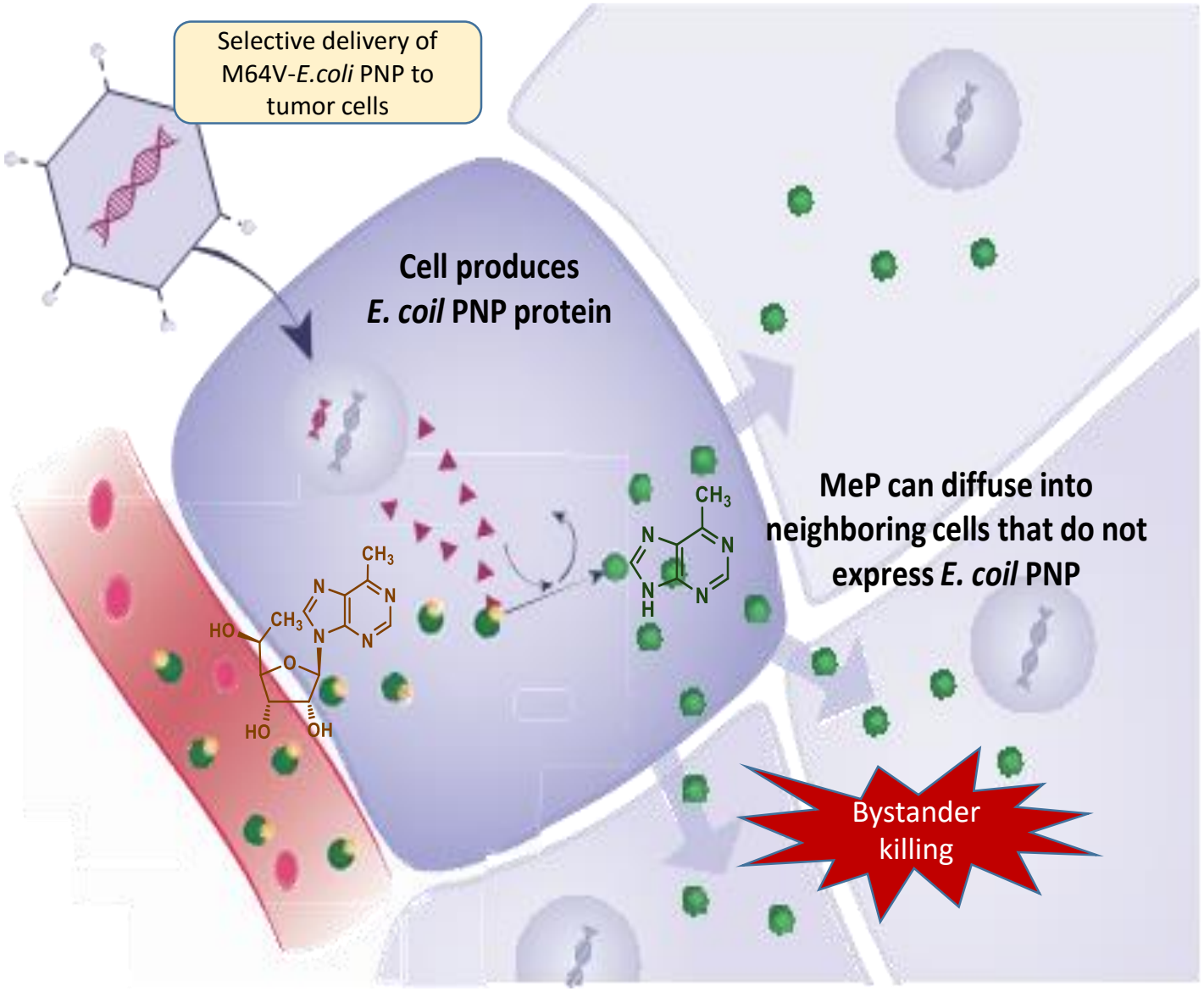
Selective delivery of M64V-*E.coli* PNP to tumor cells
Cell produces *E. coil* PNP protein
MeP can diffuse into neighboring cells that do not express *E. coil* PNP
Bystander killing

6-Methylpurine Derived Sugar Modified Nucleosides: Synthesis and *in vivo* Antitumor Activity in D54 Tumor Expressing M64V-*Escherichia coli* Purine Nucleoside Phosphorylase

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Abstract. Impressive antitumor activity has been observed with fludarabine phosphate against tumors that express *E. coli* purine nucleoside phosphorylase (PNP) due to the liberation of 2-fluoroadenine in the tumor tissue. 6-Methylpurine (MeP) is another cytotoxic adenine analog that does not exhibit selectivity when administered systemically, and could be very useful in a gene therapy approach to cancer treatment involving *E. coli* PNP. The prototype MeP releasing prodrug 9-(2-deoxy-β-D-ribofuranosyl)-6-methylpurine (**1**) [MeP-dR] has demonstrated good activity against tumors expressing *E. coli* PNP, but its antitumor activity is limited due to toxicity resulting from the generation of MeP from gut bacteria. Therefore, we have embarked on a medicinal chemistry program to identify a combination of non-toxic MeP prodrugs and non-human adenosine glycosidic bond cleaving enzymes. The two best MeP-based substrates with M64V-*E coli* PNP, a mutant which was engineered to tolerate modification at the 5'-position of adenosine and its analogues, were 9-(6-deoxy-α-L-talofuranosyl)-6-methylpurine (**3**) [methyl(talo)-MeP-R] and 9-(α-L-lyxofuranosyl)6-methylpurine (**4**) [lyxo-MeP-R]. The detailed synthesis methyl(talo)-MeP-R and lyxo-MeP-R, and the evaluation of their substrate activity with 4 enzymes not normally associated with cancer patients is described. In addition, we have determined the intraperitoneal pharmacokinetic (ip-PK) properties of methyl(talo)-MeP-R and have determined its *in vivo* bystander activity in mice bearing D54 tumors that express M64V PNP. The observed good *in vivo* bystander activity of [methyl(talo)-MeP-R/M64V-*E coli* PNP combination suggests that these agents could be useful for the treatment of cancer.

Keywords: 6-Methylpurine • 2-Fluoroadenine • Purine nucleoside phosphorylase • Prodrugs • Cancer gene therapy.

Introduction.

Suicide gene therapy is a promising strategy that attempts to limit the systemic toxicity inherent to cancer chemotherapy by selective delivery of an exogenous gene to a tumor whose expression product converts a nontoxic prodrug into a highly cytotoxic agent.[1, 2] We have developed a suicide gene therapy approach for the treatment of solid tumors that is based on selective activation of none-toxic prodrugs of toxic adenine analogs such as 2-fluoroadenine and 6-methylpurine with *E. coli* purine nucleoside phosphorylase (PNP).[3-5] *E.coli* PNP differs from human PNP in its ability to cleave the glycosidic bond of adenosine and adenosine analogs in addition to 6-oxopurine derivatives.[6, 7] Excellent *in vivo* antitumor activity has been demonstrated with this strategy against human tumor xenografts in mice using a variety of prodrugs,[8-11] including 9-[2-deoxy-β-D-ribofuranosyl]-6-methylpurine (MeP-dR; **1**), 9-[β-D-arabinofuranosyl]-2-fluoroadenine (F-araA; **2**), and 2-fluoro-2'-deoxyadenosine (F-dAdo). The best *in vivo* activity has been observed with F-araA,[12] and a phase I clinical has been initiated to evaluate its safety and efficacy.[13] However, the antitumor activity of this approach is still

limited by the inherent toxicity of the prodrugs. Although MeP-dR is much less toxic than F-araA *in vitro*, it is much more toxic to mice due to its cleavage by PNP that is expressed in intestinal bacteria,[14] which results in the systemic liberation of 6-methylpurine (MeP), which is known not to have selective *in vivo* antitumor activity.[15] In spite of this toxicity, good *in vivo* antitumor activity has been observed with MeP-dR, and it is possible that MeP prodrugs could be effectively used in a gene therapy system utilizing *E. coli* PNP, if they were not cleaved by intestinal bacteria. Therefore, we have initiated a program to change the substrate specificity of *E. coli* PNP to identify an enzyme/prodrug combination that can cleave MeP containing prodrugs that are poor substrates for the endogenous bacterial phosphorylases.[16]

Our studies have led to the identification of an *E. coli* PNP mutant (M64V) that is able to cleave numerous 5'-modified nucleoside analogs with much greater efficiency than the wild-type enzyme.[16] We have determined the crystal structure of the M64V mutant and have evaluated its activity with a few nucleoside analogs.[16] The two best MeP-based substrates were 9-(6-deoxy-α-L-talofuranosyl)-6-methylpurine (**3**) [methyl(talo)-MeP-R] and 9-(α-L-lyxofuranosyl)-6-methylpurine (**4**) [lyxo-MeP-R] (Figure 1).

Insert Figure 1

Methyl(talo)-MeP-R demonstrated good activity against D54 tumors that express M64V PNP.[16] However, cytotoxicity (MTD) of the (M64V-PNP/methyl(talo)-MeP-R) combination was observed and is attributed to the cleavage of methyl(talo)-6-MeP by unidentified bacterial enzymes.[14] To circumvent this toxicity problem, in this study, we have determined the intraperitoneal pharmacokinetic (ip-PK) properties of methyl(talo)-MeP-R and have determined the *in vivo* bystander activity of methyl(talo)-MeP-R in mice bearing D54 tumors that express M64V *E.coli*-PNP. In continuation of our efforts to identify a better (MeP-prodrug/phosphorylase) combination, we have determined the substrate activity of **3** and **4** against several other enzymes known to cleave adenosine that should not be located in the gut bacteria. The detailed synthesis of methyl(talo)-MeP-R and lyxo-MeP-R is described.

Results and Discussion

Chemistry. Synthesis of methyl(talo)-MeP-R, **3**. Vorbrüggen glycosylation of 1,2,3,5-tetra-*O*-acyl-ribofuranosides with 6-alkyl/arylpurines has been reported by us and others to selectively produce 9-(β-D-ribofuranosyl)-6-alkyl/arylpurine nucleosides.[14, 17-20] The anomeric configuration is predominantly *trans* with respect to the 1′-heterocyclic moiety and 2′-*O*-acyl group.[14, 18, 19, 21] The key intermediate needed for the coupling reaction; α-L-talofuranoside derivative **9** could be attained by the inversion of the configuration at the C-5 position of 6-deoxy-D-allofuranose derivative **6** followed by the application of the standard procedures of glycosyl donor protection and activation (Scheme 1). 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**5**) was converted in six steps to 6-deoxy-D-allofuranose derivative **6** , with minor modification of the literature procedure, in 44% total overall yield.[22, 23] Esterification of **6** under Mistunobu reaction conditions ($BzOH/PPh_3/DEAD/THF$) gave the corresponding 5-*O*-benzoate derivative **7** with inversion of the configuration at the C-5 position in 86% yield. Treatment of **7** with HCl in $MeOH/H_2O$, followed by benzoylation of the 2-hydroxyl group gave the corresponding methyl

glycoside derivative **8** in 94% yield. Acetolysis of **8** gave the corresponding 1-*O*-acetyl-α-L-talofuranose derivative **9** in 94% yield. Coupling of silylated 6-methylpurine with α-L-talofuranose derivative **9** in the presence of $SnCl_4$ in dry CH_3CN gave the corresponding 9-(2,3,5-tri-*O*-benzoyl-β-D-talofuranosyl)-6-methylpurine (**10**) in 96% yield. NOE correlation between H-1' and H-4' confirms the β-configuration of the nucleoside **10**. Irradiation at the H-1' signal showed NOE enhancement at H-4' signal (3%) and irradiation at H-4' signal showed enhancement at H-1' signal (3%). Debenzoylation of **10** with NH_3/MeOH gave **11** in 94% yield. Pd/C catalyzed hydrogenation of **11** gave 9-(6-deoxy-β-D-talofuranosyl)-6-methylpurine (**3**) in 87% yield. The β-stereochemistry of **3** was further confirmed by an NOE correlation between H-8 and H-3'. Irradiation at H-3' signal showed NOE enhancement at H-8 signal (1%) and irradiation at H-8 signal showed enhancement at H-3' signal (1%).

Insert Scheme 1

Synthesis of Lyxo-MeP-R, **4**[24]. Starting with the commercially available L-lyxose, the glycosyl donor 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-L-lyxofuranose (**12**) was prepared in three steps with minor modification of the literature procedures.[25-27] Treatment of L-lyxose with acetyl chloride in MeOH at 0 °C gave a mixture of the 1-*O*-Me-lyxofuranoside and 1-*O*-Me-lyxopyranoside derivatives in a 5:1 ratio as determined by ^{1}H-NMR. A pure β-anomer of 1-*O*-Me-lyxofuranoside was isolated by crystallization of the mixture from MeOH at -20°C. Benzoylation of methyl lyxofuranoside with BzCl in dry pyridine followed by acetolysis of the corresponding methyl 2,3,4-tri-*O*-benzoyl-lyxofuranoside derivative with Ac_2O/AcOH in the presence of conc. H_2SO_4 gave predominantly the corresponding 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-L-lyxofuranose (**12**)[27] in 89% yield. Coupling of silylated 6-methyl purine with **12** in the presence of $SnCl_4$ in dry CH_3CN to give the desired 6-MeP-lyxoside derivative **13** in 88% yield. Deprotection of **13** with NaOMe/MeOH gave then 9-(α-L-lyxofuranosyl)-6-methylpurine (**4**) in 86% yield.

Insert Scheme 2

Biology

Previous experiments have demonstrated that methyl(talo)-MeP-R had good activity against D54 tumors that express M64V PNP.[12] In order to determine the *in vivo* bystander activity of methyl(talo)-MeP-R, mice bearing tumors in which 10% of the cells expressed M64V were treated ip with methyl(talo)-MeP-R. Three days of administration of compound (days 17, 18, and 19 after tumor implantation) resulted in prolonged inhibition of tumor growth at the two highest doses of methyl(talo)-MeP-R (Figure. 2). We also determined that the terminal half-life of methyl(talo)-MeP-R was 21 minutes (Figure 3), which was a little longer than that of MeP-dR (14 minutes, calculated from the change in plasma concentration between 30 and 60 minutes).

In an effort to identify better enzymes for activating these agents, we determined the substrate activity of both methyl(talo)-MeP-R and lyxo-MeP-R against several other enzymes known to cleave the glycosidic bond of adenosine and which should not be located in gut bacteria (Table 1). Methyl(talo)-MeP-R showed approximately 8-fold less substrate activity against *T. vaginalis* PNP compared with the M64V PNP. Interestingly, moderate substrate activity was observed with lyxo-

MeP-R against *T. vaginalis* PNP and the mutant, *E. coli* M64V PNP. Poor substrate activity of both methyl(talo)-MeP-R and lyxo-MeP-R was observed with *E. coli* SAH/MTA hydrolase and *F tulorensis* PNP. Although considerable cleavage activity was observed with these enzymes, it was not better than that observed with M64V PNP, which suggests that the antitumor activity of these two prodrugs with any of these enzymes would not be superior to that seen with methyl(talo)-MeP-R and M64V.

Insert Table 1

Excellent *in vivo* bystander activity was observed with methyl(talo)-MeP-R (T-C of > 32 days at the maximally tolerated dose). In the experiment shown in Figure 2, the enzyme activity of M64V PNP using methyl(talo)-MeP-R as substrate in tumor extracts removed from mice at the initiation of treatment (day 17) was 365 ± 155 nmoles of compound cleaved/mg-hr (N = 3), whereas in previous studies[8-11] the enzyme activity of *E. coli* PNP was 40 nmoles and 11,000 nmoles of MeP-dR cleaved/mg-hr of tumor tissue, respectively. In D54 tumors in which 20% of the cells expressed low levels of *E. coli* PNP[3] there was also a good antitumor response (T-C of 26 days at the maximally tolerated dose of 67 mg/kg) , which was similar to the antitumor activity of MeP-dR (T-C of 22 days at the maximally tolerated dose of 33 mg/kg) against D54 in which 10% of the tumors expressed high levels of *E. coli* PNP.[16] Note that the dose of MeP-dR needed to be reduced in mice bearing tumors expressing high levels of *E. coli* PNP due to increased toxicity of MeP-dR in these mice. The total dose of methyl(talo)-MeP-R used in the current experiments (300 mg/kg) was approximately 3-fold greater than MeP-dR used against tumor expressing high levels of *E. coli* PNP.[16]

Insert figure 2

These results indicate that the antitumor activity of methyl(talo)-MeP-R was as good as MeP-dR, even though there was much less expression of activating enzyme in the tumor cells.[16] Although there was not a correlation of antitumor activity with *E. coli* PNP expression with MeP-dR, due to having to decrease MeP-dR administered, it is possible that better antitumor activity with methyl-(talo)-MeP-R would be observed, if D54 tumors expressed higher levels of activating enzyme (M64V).

Insert Figure 3

Conclusions.

Methyl(talo)-MeP-R and Lyxo-MeP-R were synthesized efficiently *via* Vorbrüggen glycosylation procedure. Their substrate activity with newly tested adenosine cleaving enzymes were not superior to that with the mutant enzyme M64V-*E. Coli* PNP. Although methyl(talo)-MeP-R at its maximally tolerated dose (MTD) demonstrated very good *in vivo* antitumor activity against D54 tumors that express M64V, it did not demonstrate better antitumor activity than that seen with F-araA.[11, 16, 28] Therefore, these results suggest that better MeP-prodrugs are still needed.

Efforts to identify MeP-based prodrugs with a better combination of pharmacological properties is ongoing.

Experimental Section. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Nicolet NT 300 NB spectrometer operating at 300.635 MHz (^{1}H) or 75.6 MHz (^{13}C). Chemical shifts are expressed in parts per million from tetramethylsilane. The hydrogen-decoupled ^{13}C-NMR spectra were assigned by comparison of the J_{CH} values obtained from hydrogen-coupled ^{13}C-NMR spectra. When necessary, selective hydrogen decoupling was performed in order to confirm the assignments. Ultraviolet absorption spectra were determined on Perkin-Elmer Lambda 19 spectrometer by dissolving each compound in methanol or water and diluting appropriately with 0.1*N* HCl, pH 7 buffer, or 0.1*N* NaOH. Values are in nanometers, and numbers in parentheses are extinction coefficients ($\varepsilon \times 10^{-3}$). Mass spectra were recorded on a Varian/MAT 311A double-focusing mass spectrometer in the fast atom bombardment (FAB) mode (glycerol matrix). CHN elemental analysis was carried out on Perkin-Elmer 2400 elemental analyzer. HPLC analysis was carried out on a Hewlett-Packard 1100 series liquid chromatograph with a Phenomenex Sphenclone 5 µM ODS (1) column (4.6 mm x 25 cm) with UV monitoring (254 nm). All flash column chromatography used 230-400 mesh silica gel from E. Merck. TLC was done on Analtech pre-coated (250 µm) silica gel (GF) plates.

5-*O*-Benzoyl-3-*O*-benzyl-1,2-*O*-isopropylidene-α-L-talofuranose (7). A mixture of DEAD (22 mL, 0.139 mol) and BzOH (17 g, 0.139 mol) in THF (75 mL) was added to a solution of **6** (16.4 g, 57 mmol) and Ph_3P (36.5 g, 0.139 mol) in THF (150 mL) at 0 °C under Ar atmosphere. The mixture was stirred for 10 h at rt, quenched with EtOH (10 mL) and the solvents were evaporated under reduced pressure. The residue was dissolved in EtOAc (250 mL), washed with H_2O, dried over $MgSO_4$ and evaporated. The residue was purified by a flash silica gel column (eluate; 10% EtOAc/Hexanes) to give **7** (19.5 g, 86% yield) as a pale yellow syrup. MS [FAB] *m/z* 405.1 $[M+Li]^+$, *m/z* 447.1 $[M+LiCl]^+$*; m/z* 803 $[2M+Li]^+$, ^{1}H NMR (300 MHz, $CDCl_3$): δ_H = 7.97-7.94 (2H, m, Bz), 7.54-7.37 (3H, m, Bz),7.29-7.11 (5H, m, Bn-Ar), 5.80 (1H, d, *J* = 3.5 Hz, H-1). 5.32 (1H, ddd, H-5, *J* = 3.1, *J* = 6.6, *J* = 9.7 Hz), 4.73 (1H, d, $PhCH_{2a}$, *J* = 12.1 Hz), 4.58 (1H, H-2, dd, *J* = 4.2, *J* = 3.5 Hz), 4.52 (1H, d, *J* = 12.1 Hz, $PhCH_{2b}$), 4.16 (1H, dd, H-4, *J* = 3.1, *J* = 9.0 Hz), 3.68 (1H, dd, H-3, *J* = 4.4, *J* = 9.0 Hz), 1.62 (3H, s, *i*-pr), 1.44 (3H, d, 5-CH_3, *J* = 6.6 Hz), 1.38 (3H, s, *i*-pr).

Methyl 3-*O*-benzyl-2,5-di-*O*-benzoyl-α-L-talofuranoside (8). Compound **7** (6.45 g, 16.1 mmol) was dissolved in 20% HCl in MeOH-H_2O [prepared from MeOH (150 mL), H_2O (42 mL) and AcCl (27 mL)] and the mixture was stirred for 6 h at rt. The mixture was neutralized with Et_3N and diluted with EtOAc. The whole was washed with H_2O (300 mL) and $NaHCO_3$ (300 mL). The organic phase was dried over $MgSO_4$ and evaporated. The residue was dried under vacuum for 3 hr, then dissolved in dry pyridine (150 mL) and treated with BzCl (4.2 mL, 31.5 mmol) at 0 °C under Ar atmosphere. The mixture was stirred for 30 min. at room temperature. Ice-H_2O was added to the mixture and the solvents were evaporated *in vacuo*. The residue was purified by a flash silica gel column (eluate; 5% EtOAc/Hexanes) to give **8** (7.2 g, 94% yield) as a colorless syrup. MS [FAB] *m/z* 494 $[M+NH_4]^+$, *m/z* 499 $[M+Na]^+$*; m/z* 445 $[M-OMe]^+$; ^{1}H NMR (300

MHz, $CDCl_3$): δ_H = 8.11-8.02 (4H, m, Ar), 7.59-7.40 (6H, m, Ar),7.19-7.12 (5H, m, Ar), 5.80 (1H, d, H-2, *J* = 3.74 Hz). 5.22 (1H, m, H-5), 5.04 (1H, s, H-1), 5.64 (1H, d, $PhCH_{2a}$, *J* = 11.2 Hz), 4.43 (1H, d, *J* = 11.2 Hz), 4.32-4.23 (2H, m, H-3 and H-4, *J* = 4.2, *J* = 3.5 Hz), 3.44 (3H, s, OMe), 1.62 (3H, s, *i*-pr), 1.43 (3H, d, 5-CH_3, *J* = 6.6 Hz).

9-(3-*O*-Benzyl-2,5-di-*O*-benzoyl-β-L-talofuranosyl)-6-methylpurine (10). 6-Methylpurine (5.1 g, 37.5 mmol) was suspended in a mixture of DCE (150 mL) and HMDS (150 mL) and treated with TMSCl (4.5 mL). The mixture was heated for 2 h at 80 °C whereupon a complete dissolution was observed. The solvents were evaporated and co-evaporated with toluene (100 mL x 3 times), under residue pressure, to give a white solid. A suspension of the solid residue and **9** (12.75 g, 25.5 mmol) in dry CH_3CN (600 mL) was treated with $SnCl_4$ (1*M* in CH_2Cl_2, 126 mL) at -10 °C. The mixture was stirred for 10 min whereupon the starting material was completely consumed. $NaHCO_3$ (1*M*, 450 mL) was added to the mixture, diluted with $CHCl_3$ (1 L), the organic phase was separated, dried over $MgSO_4$ and evaporated. The residue was purified by a silica gel column (eluate: 3% MeOH in $CHCl_3$) to give **10** (13.5 g, 94%) as a white foam: MS [FAB] *m/z* 579 $[M + H]^+$; UV λ_{max} pH 1: 264.3 nm; 233.7nm, pH 7: 237.1 nm, pH 13, 260.4 nm; ^1H NMR (300 MHz, $CDCl_3$): δ_H = 8.67 (1H, s, H-2), 8.22 (1H, s, H-8,), 8.11-7.12 (10H, m, Bz and Bn-Ar), 6.28 (1H, d, *J* = 2.6 Hz, H-1'), 6.22 (1H, dd, H-2', *J* = 2.4, *J* = 5.3 Hz), 5,42 (1H, m, H-5'), 4.86 (1H, dd, H-3', *J* = 5.3, *J* = 7.7 Hz), 4.69 (1H, d, CH_{2a}-Bn, *J* = 11.2 Hz), 4.69 (1H, d, CH_{2b}-Bn, *J* = 11.2 Hz), 4.56 (1H, d, CH_{2b}-Bn, *J* = 11.2 Hz), 4.36 (1H, dd, H-4', *J* = 7.9, *J* = 3.1 Hz), 2.87 (3H, s, 6-CH_3), 1.43 (3H, d, 5'-CH_3, *J* = 6.6 Hz); NOE: irradiation at H-1' signal showed enhancement at H-4' signal(3%) and irradiation at H-4' signal showed enhancement at H-1' signal (3%); Anal. Calcd. for $C_{30}H_{30}N_4O_6 \cdot 0.5\ H_20$: C, 67.67; H, 5.42; N, 9.42. Found: C, 67.69; H, 5.46; N, 9.29.

9-(3-*O*-Benzyl-α-L-talofuranosyl)-6-methylpurine (11). A solution of **10** (6 g, 10.4 mmol) was dissolved in saturated NH_3/MeOH (130 mL) and the mixture was kept stirring at rt for 2 days. The solvent was evaporated and the residue was purified over a silica gel column (eluate: 7 % MeOH in $CHCl_3$) to give **11** (3.6 g, 96%) as a white glassy solid: ^1H NMR (300 MHz, $CDCl_3$): δ_H = 8.80 (1H, s, H-2), 8.79 (1H, s, H-8), 7.42-7.31 (5H, m, Bn-Ar), 6.08 (1H, d, H-1', *J* = 6.1 Hz), 5.64 (1H, d, 2'-OH, *J* = 6.4 Hz, exchangable with D_2O), 5.17 (1H, d, *J* = 6.3 Hz, exchangable with D_2O, 5'-OH), 4.78 (1H, m, H-2'), 4.76 (1H, d, CH_{2a}-Bn, *J* = 12.2 Hz), 4.63 (1H, d, *J* = 12.2 Hz, CH_{2b}-Bn), 4.09 (1H, dd, H-3', *J* = 3.4, *J* = 4.7 Hz), 4.02(1H, t, H-4', *J* = 3.3 Hz), 3.82 (1H, m, H-5'), 2.47(3H, s, 6-CH_3), 1.13 (3H d, 5'-CH_3, *J* = 6.5 Hz); NOE irradiation at H-1' signal showed enhancement at H-4' signal (1%) and irradiation at H-4' signal showed enhancement at H-1' signal (2%); UV λ_{max} pH 1: 263.7 nm; 233.7 nm, pH 7: 244.1 nm, 260.2; pH 13, 260.7 nm; MS [FAB] *m/z* 371 $[M + H]^+$, 377.1 $[M + Na]^+$; Anal. Calcd. for $C_{19}H_{22}N_4O_4 \cdot 0.5\ H_20$: C, 60.46; H, 5.99; N, 14.73. Found: C, 60.29; H, 6.11; N, 14.77.

9-(6-Deoxy-α-L-talofuranosyl)-6-methylpurine (3). A mixture of **11** (2.3 g, 6.1 mmol) and 10% Pd/C (0.5 g) in EtOH (95%, 100 mL) was stirred for 6 h at room temperature under H_2 atmosphere. The mixture was filtered over Celite pad and the filtrate was evaporated under reduced pressure. The residue was purified over a silica gel column (eluate: 10 % MeOH in $CHCl_3$) to give **3** (1.5 g, 87%) as a white solid: MS [FAB] *m/z* 280.9 $[M + H]^+$; UV λ_{max} pH 1: 263.9 nm; 233.8 nm, pH 7: 244.5 nm, 260.9; pH 13; 260.8 nm; HPLC [100%; RT 14.918 min; 0.01*M* $NH_4H_2PO_4$: MeOH (85

; 15); 20 min linear gradient from 10-90% B]; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 8.79 (1H, s, H-2), 8.78 (1H, s, H-8), 6.02 (1H, d, H-1', *J* = 5.7 Hz), 5.50 (1H, d, 2'-OH, *J* = 5.9 Hz, exchangable with D_2O), 5.18 (1H, d, 3'-OH, *J* = 5.1 Hz, exchangable with D_2O), 5.09 (1H, d, 5'-OH, *J* = 5.9 Hz, exchangable with D_2O), 4.54 (1H, m, H-2'), 4.14 (1H, m, H-3'), 3.85-3.81 (2H, m, H4' and H-5'), 2.74 (1H, s, 6-CH_3), 1.14 (3H, d, 5'-CH_3, *J* = 6.15 Hz); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C = 158.62 (C-6), 151.46 (C-2), 149.93 (C-4), 144.35 (C-8), 133.07 (C-5), 92.08 (C-4 `), 87.23 (C-1'), 73.7 (C-2'), 69.78 (C-3'), 69.67 (C-5'), 26.42 (5'-CH_3), 26.08 (5'-CH_3). NOE: irradiation at H-3' signal showed enhancemnet at H-8 signal (1%) and irradiation at H-8 signal showed enhancemnet at H-3' signal (1%); Anal. Calcd. for $C_{12}H_{16}N_4O_4$: C, 42; H, 75; N, 19.98. Found: C, 51.37.; H, 5.73; N, 19.74.

1-*O*-Acetyl-2,3,5-tri-*O*-benzoyl-α-L-lyxofuranose (12).[27] L-Lyxose (5 g, 33.3 mmol) was dissolved in dry MeOH (40 mL) and treated with conc. H_2SO_4 (40 μL) at 0 °C under Ar. atmosphere and the mixture was stirred for 72 h at rt. Pyridine (2 mL) was added and the solvents were evaporated and coevaporated with toluene under reduced pressure. The residue was purified by a silica gel column (eluate: 7% MeOH in $CHCl_3$) to give Methyl α-L-lyxofuranoside[26] (3.34 g, 61 % yield) as a mixture of α and β anomers, which was crystalized from cold MeOH: ^{1}H NMR (300 MHz, DMSO-d_6,) δ_H = 4.85 (1H, d, H-1, *J* = 3.73 Hz). 4.23 (1H, m, H-3), 4.15 (1H, dt, H-4, *J* = 4.2, *J* = 6.8 Hz), 4.02 (1H, dd, H-2, *J* = 3.7, *J* = 4.8 Hz), 3.74 (1H, dd, H-5^a, *J* = 4.3, *J* = 11.9 Hz), 3.64 (1H, dd, H-5b, *J* = 6.8, *J* = 11.9 Hz), 3.24 (3H, s, OCH_3). Benzoyl chloride (6.3 mL, 54.9 mmol) was added to a solution of 1-*O*-Methyl-α-L-lyxofuranose (2 g, 12.2 mmol) in dry pyridine (50 mL) at 0 °C. The mixture was stirred for 30 min. at 0 °C, MeOH (2 ml) was added and the mixture was stirred further for 10 min. The solvents were evaporated and the residue was partitioned between EtOAc and H_2O. The organic phase was dried ($MgSO_4$) and evaporated. The residue was purified by a flash silica gel column (eluate: 25% EtOAc in hexanes) to give Methyl 2,3,5-tri-*O*-benzoyl-α-L-lyxofuranoside (4.1 g, 73% yield) as a white solid: MS [FAB] *m/z* 477.5 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H = 7.99-7.96 (6H, m, *O*-Bz), 7.53-7.47 (3H, m, *p*-Bz), 7.40-7.28 (6H, m, *m*-Bz), 6.03 (1H, t, H-3, *J* = 5.6 Hz), 5.63 (1H, dd, H-2, *J* = 1.43, *J* = 5.3 Hz), 5.23 (1H, d, H-1, *J* = 1.43 Hz), 4.84 (1H, dd, H-4, *J* = 5.9, *J* = 6.1 Hz), 4.72-4.60 (2H, m, $H5_{a,b}$), 3.49 (3H, s, O-CH_3). Methyl 2,3,5-tri-*O*-benzoyl-α-L-lyxofuranoside (1.2 g, 2.52 mmol) was dissolved in AcOH (36 mL), Ac_2O (3.6 mL) and the mixture was cooled to 0 °C. conc. H_2SO_4 (150 μL) was added dropwise to the mixture at 0 °C over 30 min. The mixture was stirred for 2 h at rt. The mixture was pured into a cold saturated $NaHCO_3$ solution and diluted with EtOAc. The organic phase was separated, dried over ($MgSO_4$) and evaporated. The residue was purified by a flash silica gel column (eluate; 30% EtOAc/Hexanes) to give **12** (1.14 g, 89% yield) as a white solid: MS [FAB] *m/z* 505 $[M + H]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H = 7.97-7.88 (6H, m, *o*-Bz), 7.53-7.52 (3H, m, *p*-Bz's), 7.40-7.32 (6H, m, *m*-Bz's), 6.50 (1H, d, H-1, *J* = 1.9 Hz), 6.09 (1H, dd, H-3, *J* = 5.2, *J* = 5.8 Hz), 5.78 (1H, dd, H-2, *J* = 1.9, *J* = 5.2 Hz), 4.94 (1H, dd, H-4, *J* = 5.8, *J* = 6.0 Hz), 4.67- 4.65 (2H, m, $H5_{a,b}$), 2.17 (3H, s, CH_3).

9-(2,3,5-tri-*O*-Benzoyl-α-L-lyxofuranosyl)-6-methylpurine (13). 6-Methylpurine (0.66 g, 4.8 mmol) was suspended in a mixture of DCE (30 mL) and HMDS (6 mL) and treated with TMSCl (0.6 mL). The mixture was heated for 3 h at 80 °C whereupon a complete dissolution was

observed. After cooling down to room temperature, the solvents were evaporated and co-evaporated with toluene (60 mL x 3 times), under reduced pressure to give a white solid. Compound **12** (1.14 g, 2.22 mmol) in dry CH_3CN (40 mL) was added to the silylated 6-MeP, the mixture was then treated with $SnCl_4$ (1*M* in CH_2Cl_2, 11.1 mL) at -10 °C. The mixture was stirred for 4 h whereupon the starting material was completely consumed. Cold $NaHCO_3$ (1*M*, 140 mL) was added to the mixture, diluted with $CHCl_3$ (150 mL), the organic phase was separated, dried over $MgSO_4$ and evaporated. The residue was purified by a silica gel column (eluate: 1.5:1 EtOAc : hexanes) to give **13** (1.17 g, 88%) as a white foam: MS [FAB] *m/z* 579 $[M + H]^+$; UV λ_{max} pH 1: 265.1 nm; 232.7 nm, pH 7: 245.6 nm, pH 13, 260.6 nm;224.2 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.91 (1H, s, H-2), 8.15 (1H, s, H-8), 8.07-7.25 (15H, m, Bz), 6.78 (1H, t, H-2', *J* = 5.2 Hz), 6.44-6.40 (2H, m, H-2' and H-3'), 5.50 (1H, m, H-4'), 4.80 (1H, dd, H-5'$_a$, *J* = 8.94, *J* = 11.8 Hz), 4.71 (1H, dd, H-5'$_b$, *J* = 5.2, *J* = 11.8 Hz), 2.88 (3H, s, 6-CH_3,); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 166.06 (C=O, Bz), 165.13 (C=O, Bz), 165.03 (C=O, Bz), 159.95 (C-6), 152.45 (C-2), 150.33 (C-4), 143.12 (C-8), 133.98 (C-5), 133.75 (C-Ph), 133.68 (C-Ph), 133.21 (C-Ph), 129.79 (C-Ph), 129.72 (C-Ph), 129.30 (C-Ph), 128.73 (C-Ph), 128.62 (C-Ph), 128.40 (C-Ph), 128.33 (C-Ph), 128.15 (C-Ph), 88.03 (C-1'), 79.47 (C-4'), 74.88 (C-2'), 72.40 (C-3'), 62.33 (C-5'), 19.52 (6-CH_3); Anal. Calcd. for $C_{32}H_{26}N_4O_7$: C, 66.43; H, 4.53; N, 9.68. Found: C, 66.13; H, 5.66; N, 9.08.

9-(α-L-Lyxofuranosyl)-6-methylpurine (4). To a solution of **13** (0.54 g, 1.59 mmol) in dry MeOH (15 mL) was added NaOMe (1*M* soln., 0.8 mL) dropwise at 0 °C. The mixture was stirred for 2 h at rt, then neutralized with Amperlyst 50 (H^+, weakly acidic). The mixture was filtered off and the filtrate was evaporated under reduced pressure. The residue was purified by a flash silica gel column (eluate: 10%MeOH in $CHCl_3$) to give 4 (0.12 g, 86%) as a white solid: MS [FAB] *m/z* 267 $[M + H]^+$; UV λ_{max} pH 1: 264.5 nm; pH 7: 260.5 nm, pH 13, 260.6 nm; ^{1}H NMR (300 MHz, DMSO-d_6) δ = 8.81 (1H, s, H-2), 8.79 (1H, s, H-8), 6.00 (1H, d, H-1', *J* = 7.3 Hz), 5.53 (1H, d, 2'-OH, *J* = 6.77 Hz, exchangable with D_2O), 5.22 (1H, d, 3'-OH, *J* = 4.1 Hz, exchangable with D_2O), 5.05 (1H, m, H-2', *J* = 4.18, *J* = 7.3 Hz), 4.68 (1H, t, 5'-OH, *J* = 5.6 Hz, exchangable with D_2O), 4.54 (1H, m, H-4'), 4.21 (1H, m, H-3'), 3.68 (1H, m, H-5'a, *J* = 5.3, *J* = 11.43 Hz), 3.55 (1H, m, H-5'b, *J* = 6.6, *J* = 11.43 Hz,), 2.74 (3H, s, 6-CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ = 158.30 (C-6), 151.69 (C-2), 150.28 (C-4), 144.82 (C-8), 133.1 (C-5), 87.73 (C-1'), 82.85 (C-4'), 74.67 (C-2'), 70.87 (C-3'), 59.84 (C-5'), 19.11 (6-CH_3); Anal. Calcd. for $C_{11}H_{14}N_4O_4$·0.2 CH_3OH: C, 49.34; H, 5.47; N, 20.55. Found: C, 49.43; H, 5.47; N, 20.56.

Methods for Biological Section.

***In vivo* studies**. Parental and M64V PNP expressing D54MG (human glioma) tumor cells were mixed so that 10% of the cells expressed M64V PNP. This mixture was injected subcutaneously (2 x 10^7 total cells) into the flanks of nude mice (nu/nu) purchased from Charles River Laboratories (Wilmington, MA, USA). When the tumors reached the appropriate size (approximately 300 mg), the mice were treated with methyl(talo)-MeP-R. The compound was administered in the peritoneal cavity 5 times a day (every 2 hours) for 3 consecutive days at doses of 5, 10, or 20 mg/kg. There were 6 mice per treatment group. The effect of compound on tumor size was determined by measuring the tumors twice a week with calipers. Tumor weight was calculated from the formula: [length (mm) x width^2 (mm)]/2 = mg, assuming unit density and assuming that the tumor takes the general shape of a prolate ellipsoid. In all *in vivo* experiments, mice were

examined daily for evidence of mortality or other gross clinical signs. All procedures were performed in accordance with a protocol that was approved by the IACUC of Southern Research Institute.

Enzyme assay and HPLC analysis of purine nucleosides and bases. Enzymes were incubated with various substrates in 1000 µl reactions containing 50 mM potassium phosphate (pH 7.4), 100 µM of substrate, and an appropriate amount of enzyme so that linear increase in product formation could be followed over time. After incubation for 0, 0.25, 0.5, 1, and 2 hours at 25°C, 150 µl of the reaction was removed and mixed with 150 µl of water, and the reaction was stopped by boiling. The precipitated proteins were removed by filtration (0.2 µm syringe filter), and the sample was injected onto a 5 µm BDS Hypersil C-18 column (150 x 4.6 mm) (Keystone Scientific Inc., State College, PA). The mobile phase was a 50 mM ammonium dihydrogen phosphate buffer (pH 4.5) containing acetonitrile (flow rate of 1 ml/minute). The appropriate amount of acetonitrile (1.25 to 5%) was determined for each compound to optimize separation of substrate and product. The nucleosides and their respective bases were detected as they eluted from the column by their absorbance at their 254 nm. The percent conversion of substrate to product was calculated and the specific activity was determined by dividing the number of nmoles of product formed by the mg protein and the time of incubation.

Determination of plasma concentration of MeP-dR and methyl(talo)-MeP-R. Groups (4 mice/group) of nontumored animals were euthanized at various times (5, 15, 30, 60, and 120 minutes) after a single intraperitoneal injection of 100 mg/kg of [^{3}H]-methyl(talo)-MeP-R or 67 mg/kg [^{3}H]MeP-dR. The plasma was collected into heparinized tubes and frozen until the amount of methyl(talo)-MeP-R or MeP-dR in each plasma sample could be determined using reverse phase HPLC analysis as described above. The plasma samples were centrifuged through a Centrifree Ultrafiltration Device (Millipore) prior to analysis by HPLC.

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Figure Captions

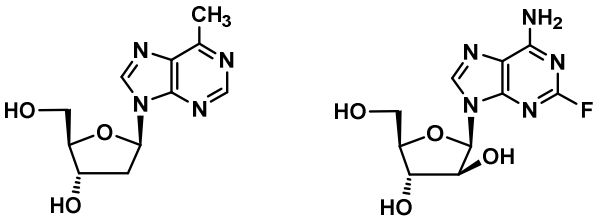
Figure 1. 6-Methylpurine and 2-fluoroadenine based prodrug structures.
Figure 2. Effect of methyl(talo)-MeP-R on D54 tumors in which 10% of the cells express M64V-PNP.
Figure 3. Plasma concentrations after IP administration of methyl(talo)-MeP-R and MeP-dR.

Table 1. Activity of sugar modified nucleosides with various phosphorylase enzymes.

Compd.	Human PNP (nmol/mg/hr)	Human MTAP (nmol/mg/hr)	*E. coli* PNP (nmol/mg/hr)	*E. coli* M64V-PNP (nmol/mg/hr)	*T. vag* PNP (nmol/mg/hr)	*S. sul* MTAP (nmol/mg/hr)	SAH/MTA Hydrolyase (nmol/mg/hr)	*F. tul* PNP (nmol/mg/hr)
Adenosine	-	596	398,000*	-	501,000	57,000	21,00	2,100
Inosine	391,000*	-	342,000*	-	154,000	84,000	-	2000
MeP-dR	35	<1	528,000*	593,000	484,000	12,000	30	3,900
MeP-R	12	-	96,000*	176,000	155,000	4,000	290	4,800
Lyxo-MeP-R	-	<1	218	10,000**	10,000	1,400	36	180
methyl(talo)-MeP-R	<1	4	915	86,000**	8,400	1500	51	100

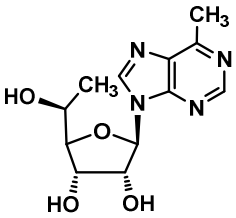
Enzymes were incubated at 25°C with 100 μM of each compound, 50 mM potassium phosphate (pH 7.4), and an appropriate amount of enzyme to measure a linear rate of cleavage. Substrate activity was measured using reverse phase HPLC to separate product from substrate [8]. The enzyme concentration was changed based on substrate activity and the lower limit for detection of activity is 1 nmoles/mg/hr. Each value shown is the average of at least two experiments which were in good agreement. All enzymes were recombinants except for tularemia PNP which was isolated from crude extract from tularemia. M64V PNP was prepared as described [12]. *T. vaginalis* PNP, *S. solfataricus* MTAP, and *E. coli* SAH/MTA hydrolase was obtained for Dr. Steve Ealick at Cornell University. * Reference [5]. **Reference [12,16].

Figure 1. Prodrug structures for 6-methylpurine and 2-fluoroadenine

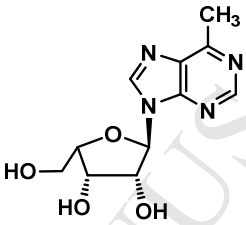


1; MeP-dR

2; F-araA

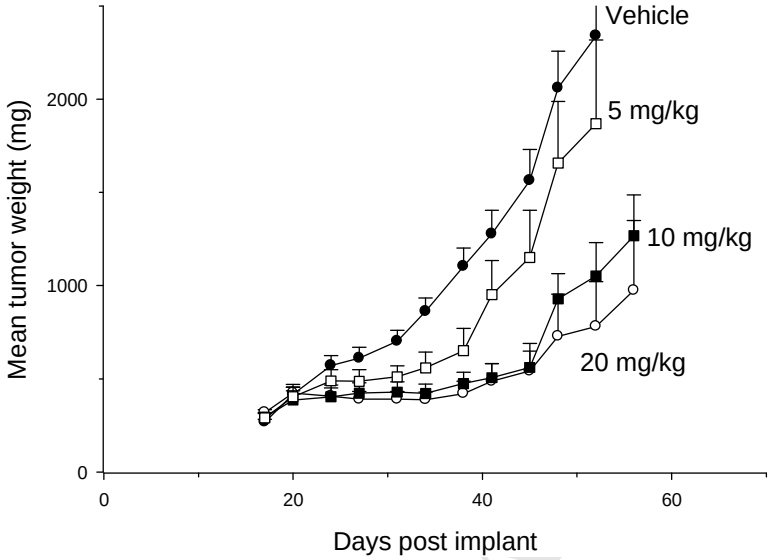


3; Methyl(talo)-MeP-R



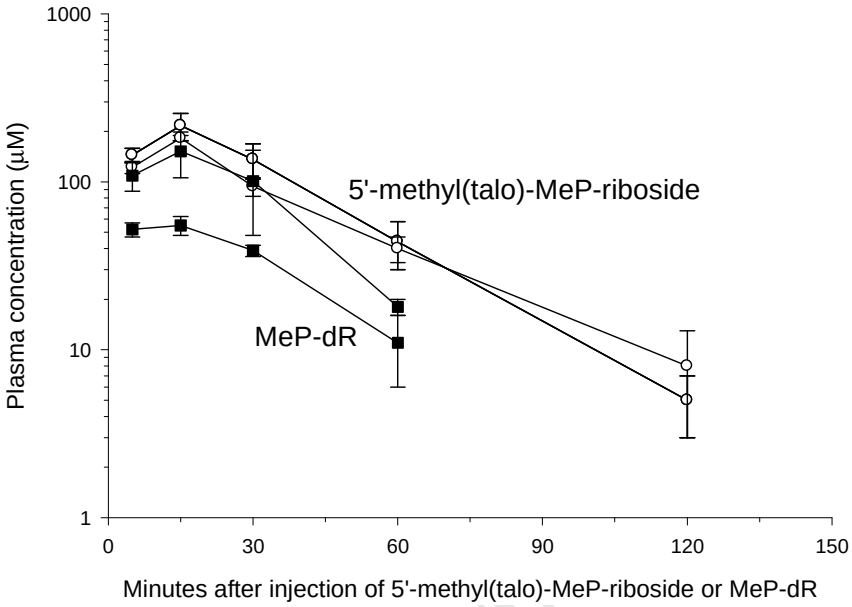
4; Lyxo-MeP-R

Figure 2. Effect of methyl(talo)-MeP-R on D54 tumors in which 10% of the cells express M64V PNP.



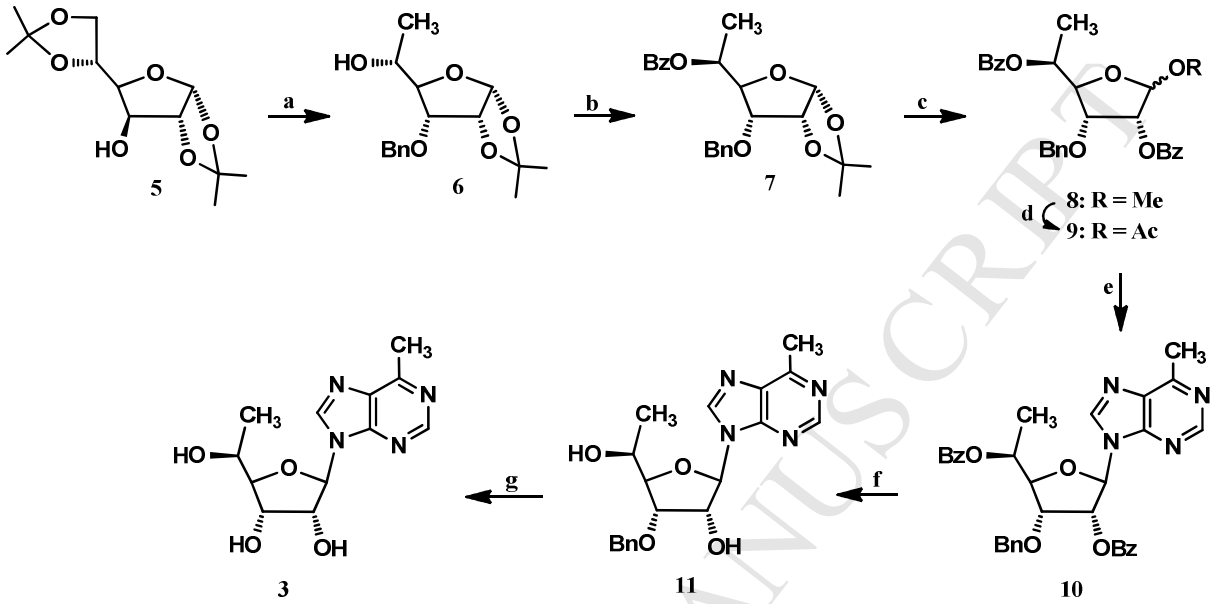
Wild-type D54 tumor cells were mixed with D54 tumor cells that had been transduced with the M64V PNP gene so that 10% of the mixture contained cells that expressed M64V PNP. This 90/10 mixture was injected sc into the flanks of nude mice. Mice were treated with methyl(talo)-MeP-R at the doses shown in the figure 5 times daily (every 2 hours) for 3 consecutive days beginning on day 17 when tumors were approximately 300 mg. Values shown are the mean and SEM of 6 measurements (10 in the vehicle treated group). The experiment has been repeated with similar results.

Figure 3. Plasma concentrations after IP administration of methyl(talo)-MeP-R and MeP-dR.



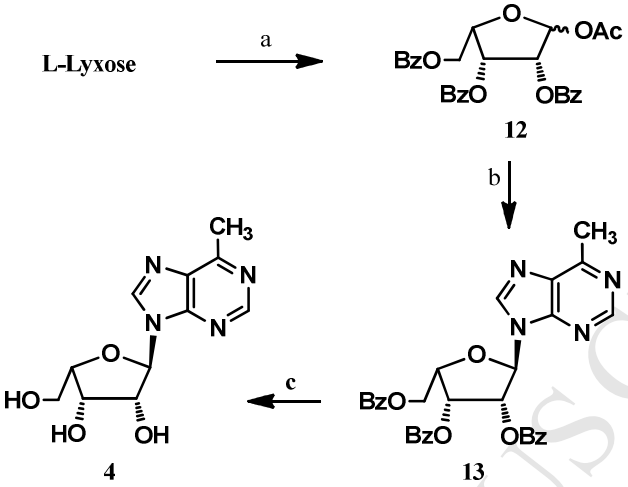
Mice were injected ip with 100 mg/kg [^{3}H]5'-methyl(talo)-MeP-riboside or 67 mg/kg [^{3}H]MeP-dR . Four mice were sacrificed 5, 15, 30, 60, and 120 minutes after injection of drug, and the plasma was collected and frozen until the amount of 5'-methyl(talo)-MeP-riboside (open circles) or MeP-dR (filled squares) could be determined using reverse phase HPLC analysis. The figure shows the result of two experiments for each compound. The results are the mean and standard deviation.

Scheme 1[a]



[a]Reagents and conditions. a) ref. 11. b) DEAD, TPP, BzOH, THF, 0 °C, 10 h, 86%; c) 20% HCl/MeOH-H_2O, 6 h , rt, then BzCl, dry pyr., 30 min, rt, 94%; d) Ac_2O, AcOH, conc. H_2SO_4, 4 h, rt, 94%; e) 6-Mep, HMDS, DCE, TMSCl, 3 h, 80 °C, then $SnCl_4$, CH_3CN, -10 °C, 10 min, 96%; f) NH_3/MeOH, 48 h, rt, 94%; g) 10% Pd/C , 95% EtOH, H_2, 6 h, rt, 87%.

Scheme 2[a]



[a]Reagents and conditions. a) i) conc. H_2SO_4, MeOH, 72 h, rt; ii) BzCl, dry Pyr., 30 min, 0 °C; iii) Ac_2O, AcOH, conc. H_2SO_4, 0 °C, 30 min, 39.6%; b) 6-MeP, HMDS, DCE, TMSCl, 3 h, 80 °C, then $SnCl_4$, CH_3CN, -10 °C, 40 min, 88%; c) NaOMe, MeOH, 2 h, rt, 86%.

Highlights

- Methyl(talo)-MeP-R; **3** and lyxo-MeP-R; **4** were synthesized and evaluated as potential prodrugs.
- The substrate activity **3** and **4** were evaluated with several phosphorylase enzymes.
- In conjunction with M64V-*E. coli* PNPm, **3** showed good *in vivo* bystander activity.