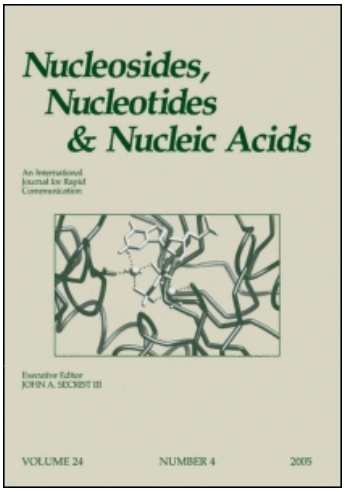

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Parker, William B. , Allan, Paula W. , Ealick, Steve E. , Sorscher, Eric J. , Hassan, Abdalla E. A. , Silamkoti, A. V. , Fowler, Anita T. , Waud, William R. and Secrist III, John A.(2005) 'Design and Evaluation of 5'-Modified Nucleoside Analogs as Prodrugs for an *E. coli* Purine Nucleoside Phosphorylase Mutant', Nucleosides, Nucleotides and Nucleic Acids, 24: 5, 387 — 392
To link to this Article: DOI: 10.1081/NCN-200059807
URL: http://dx.doi.org/10.1081/NCN-200059807

Nucleosides, Nucleotides, and Nucleic Acids, 24 (5–7):387–392, (2005)

ISSN: 1525-7770 print/ 1532-2335 online
DOI: 10.1081/NCN-200059807

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DESIGN AND EVALUATION OF 5′-MODIFIED NUCLEOSIDE ANALOGS AS PRODRUGS FOR AN *E. COLI* PURINE NUCLEOSIDE PHOSPHORYLASE MUTANT

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▫ *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. The biological activity of the three best substrates of this mutant (9-[6-deoxy-α-L-talofuranosyl]-6-methylpurine (methyl(talo)-MeP-R), 9-[6-deoxy-α-L-talofuranosyl]-2-F-adenine, and 9-[α-L-lyxofuranosyl]-2-F-adenine) were evaluated so that we can optimally utilize these compounds. Our results indicated that the mechanism of toxicity of methyl(talo)-MeP-R to mice was due to its cleavage to MeP by a bacterial enzyme, and that the toxicity of the two F-Ade analogs was due to their cleavage to F-Ade by mammalian methylthioadenosine phosphorylase.*

INTRODUCTION

E. coli purine nucleoside phosphorylase (PNP) differs from human PNP in its ability to cleave adenosine and its analogs[1,2] and is being developed as a suicide gene therapy approach for the treatment of solid tumors.[3,4] Although excellent in vivo antitumor activity has been demonstrated with this strategy against human tumor xenografts in mice,[5–8] the antitumor activity is still limited by the toxicity of the prodrugs (9-[2-deoxy-β-D-ribofuranosyl]-6-methylpurine (MeP-dR); 2-F-2′-deoxyadenosine (F-dAdo), and 9-[β-D-arabinofuranosyl]-2-F-adenine (F-araA)). We have shown that the in vivo toxicity of MeP-dR is due to its cleavage by PNP that is expressed in intestinal bacteria,[9] which results in the liberation of MeP, a

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compound that does not have selective antitumor activity when administered systemically.[10]

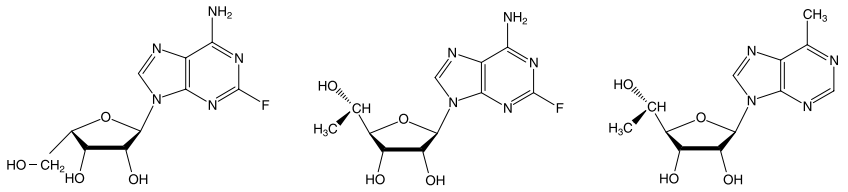
Because of this toxicity, we have initiated a program to change the substrate specificity of *E. coli* PNP to identify an enzyme/prodrug combination that can cleave prodrugs that are poor substrates for the endogenous bacterial phosphorylases. Our studies have led to the identification of an *E. coli* PNP mutant (M64V) that is able to cleave numerous 5′-modified nucleoside analogs with much greater efficiency than the wild-type enzyme. We have determined the crystal structure of the M64V mutant and have evaluated its activity with a few nucleoside analogs.[9]

RESULTS AND DISCUSSION

The three best M64V substrates (Figure 1 and Table 1), 9-[6-deoxy-α-L-talofuranosyl]-6-methylpurine (methyl(talo)-MeP-R), 9-[6-deoxy-α-L-talofuranosyl]-2-F-adenine (methyl(talo)-F-Ado), and 9-α-L-lyxofuranosyl-2-F-adenine (lyxo-F-Ade), were prepared in gram quantities by the RAND mechanism at the National Cancer Institute so that they could be evaluated for in vivo activity. Methyl(talo)-MeP-R and lyxo-F-Ade at their maximally tolerated dose (MTD) demonstrated anti-tumor activity against D54 tumors that express M64V (data not shown). However, neither compound demonstrated better antitumor activity than did the original prodrugs with the wild-type enzyme.

Methyl(talo)-MeP-R was not toxic to any of the human cell lines tested (Table 2), and its maximally tolerated dose (MTD) in mice was approximately 500 mg/kg (given daily for 3 consecutive days), which was approximately 5 times the MTD of MeP-dR. The difference in the MTD between MeP-dR and methyl(talo)-MeP-R (5-fold) was much less than the difference in the activity of these compounds with *E. coli* PNP (600-fold), which suggested that a mechanism of toxicity not associated with *E. coli* PNP has become limiting for methyl(talo)-MeP-R.

In contrast, the F-Ade analogs demonstrated moderate to significant toxicity to the various cell lines (Table 2). Methyl(talo)-F-Ado had IC_{50}s of less than 1 μM in 4 of the 8 cell lines evaluated. The IC_{50}s of F-Ade and F-Ado were included in the table for comparison purposes. The MTD of lyxo-F-Ade was 20 mg/kg (given 5 times a day [every 2 h] for 3 consecutive days), which was the same as that for



lyxo-F-Ade methyl(talo)-F-Ado methyl(talo)-MeP-R

FIGURE 1 Structures of lyxo-F-Ade, methyl(talo)-F-Ado, and methyl(talo)-MeP-R.

TABLE 1 Enzyme Activity of *E. coli* PNP and M64V

Substrate	*E. coli* PNP	M64V (nmoles/mg/hr)
MeP-dR	530,000	590,000
F-dAdo	440,000	1,600,000
F-araA	1,200	1,000
Methyl(talo)-MeP-R	920	86,000
Methyl(talo)-F-Ado	34,000	1,400,000
Lyxo-F-Ade	7,800	240,000

The enzymes were incubated with 100 μM of each compound and the rate of cleavage was determined by HPLC separation of base from nucleoside. Each number is the average of at least two determinations.

F-dAdo. Methyl(talo)-F-Ado was also toxic to mice with a MTD of 5 mg/kg (given on the same schedule as lyxo-F-Ade).

To evaluate the role of intestinal bacteria in the toxicity of these agents, the MTD of these compounds was determined in mice that had been treated with non-absorbable antibiotics.* Only the MTD of methyl(talo)-MeP-R was increased in these mice, which indicated that an enzyme expressed in intestinal bacteria was able to cleave methyl(talo)-MeP-R to MeP. Since the F-Ade analogs were toxic to cells in vitro and alteration of intestinal bacteria did not affect their toxicity to mice, the toxicity of these two agents was due to their activation to toxic forms by mammalian enzymes.

5′-Methylthioadenosine phosphorylase (MTAP) is another phosphorylase, which is found in most mammalian cells, and is known to cleave 5′-modified adenine analogs. Since it is possible that this enzyme could be responsible for cleaving lyxo-F-Ade and methyl(talo)-F-Ado to F-Ade in human cells, these compounds were evaluated as substrates for human MTAP (Table 3). Both of the MeP analogs tested were very poor substrates for human MTAP. Very little substrate activity was detected with methyl(talo)-MeP-R, which is consistent with the observation that this compound was not very toxic to human cells. However, lyxo-F-Ade and methyl(talo)-F-Ado were cleaved at 5 and 23%, respectively, of the rate of methylthioadenosine by human MTAP, which indicated that a significant amount of F-Ade would be generated in mice treated with these two compounds. Since F-Ade is a very toxic compound, this result is consistent with the potent cytotoxicity of these two agents.

The 5′-triphosphate of 2-F-adenosine (F-ATP) was detected in CAKI-I cells (cells that express MTAP) that were treated with lyxo-F-Ade (Table 4). Incubation in the presence of 5′-methylthio-immucillin-adenine (MT-immucillin-A), a potent inhibitor of human MTAP, resulted in decreased formation of F-ATP and

*Antibiotic treatment was as follows: For the first week the following agents were added to the drinking water: neomycin (5 g/L), streptomycin (5 g/L), bacitracin (5 g/L), and pimaricin (0.1 g/L). For the second and third weeks the concentration of neomycin, streptomycin, and bacitracin were reduced to 2.5 g/L and pimaricin was maintained at 0.1 g/L. Initiation of prodrug treatment began at the end of two weeks of antibiotic therapy.

TABLE 2 In vitro Cytotoxicity of 5′-Modified Nucleoside Analogs

	Cell line							
Compound	SNB-7	DLD-1	CEM	H23 IC_{50} (μM)	ZR-75-1	LOX	PC-3	CAKI-1
F-Ade	1.2 ± 0.5	0.54 ± 0.09	0.11 ± 0.02	0.19 ± 0.09	0.32 ± 0.02	0.25 ± 0.16	0.32 ± 0.05	0.08 ± 0.08
F-Ado (2)	0.93	0.32	0.17	0.12	0.30	0.53	0.24	0.06
Methyl(talo)-F-Ado	5 ± 0.7	4.8 ± 1.0	13 ± 5	0.43 ± 0.14	0.82 ± 0.18	26 ± 12	0.45 ± 0.09	0.061 ± 0.003
Lyxo-F-Ade	50 ± 19	45 ± 23	27 ± 18	8 ± 6	12 ± 5	32 ± 12	9 ± 5	1.9 ± 1.2
Methyl(talo)-MeP-R (2)	> 210	> 210	> 210	> 210	> 210	> 210	> 210	> 210

Cells were incubated with various concentrations of the compounds shown in the table. The effect of compound on cell growth was determined using standard procedures. The IC_{50} is the concentration of drug required to inhibit cell growth by 50%. The numbers presented are the mean ± SD of at least 3 separate determinations except where only 2 measurements were made.

TABLE 3 MTAP Activity

Substrate	Specific activity (nmoles/mg/hr)
Methylthioadenosine	149,000
MeP-dR	<1
Methyl(talo)-MeP-R	4
F-dAdo	600
F-araA	<1
Lyxo-F-Ade	7,400
Methyl(talo)-F-Ado	34,000

MTAP was incubated with 100 μM of each compound and the rate of cleavage was determined by HPLC separation of base from nucleoside. Each number is the average of at least two determinations. The lower limit for detection of activity is approximately 1 nmoles/mg/hr.

prevented the cytotoxicity of lyxo-F-Ade. These results indicated that the toxicity of lyxo-F-Ade to human cells was due to its cleavage by human MTAP to F-Ade. Similar metabolism studies have not been done with methyl(talo)-F-Ado, but given its good activity with human MTAP, it is likely that its mechanism of toxicity is similar to that of lyxo-F-Ade.

Methyl(talo)-MeP-R and lyxo-F-Ade were labeled with tritium[†] so that their metabolism could be evaluated. There was very little metabolism of either compound in CEM cells (a cell line that does not express MTAP), which indicated that this compound was a very poor substrate for mammalian kinases (data not shown). The compounds were phosphorylated at a rate that was 50,000 times less

[†]Tritium exchange was done by Moravek Biochemicals (Brea, California).

TABLE 4 Effect of MT-Immucillin-A on the Metabolism and Cytotoxicity of Lyxo-F-Ade in CAKI-I Cells

Treatment	ATP	F-ATP (nmoles)	IC_{50} (μM)
No treatment	23	0	–
Lyxo-F-Ade	26	3.3	0.4
Lyxo-F-Ade + MT-immucillin-A	22	0.2	21

CAKI-I were incubated with no drugs, 100 μM lyxo-F-Ade, or 100 μM lyxo-F-Ade plus 10 μM MT-immucillin-A for 4 h. The cells were removed from the flasks and an acid-soluble extract was obtained as described previously.[12] The extract was analyzed by strong anion exchange chromatography and the amount of ATP and F-ATP were determined by their absorbance at A_{260}. The effect of the compounds on cell growth was also determined using standard procedures. The IC_{50} is the concentration of lyxo-F-Ade required to inhibit cell growth by 50% after 72 h of incubation.

than that of adenosine, and their phosphorylation was inhibited by iodotubercidin, a potent inhibitor of adenosine kinase. The toxicity of lyxo-F-Ade to CEM cells (IC_{50} of 27 μM) could be explained by the presence of F-Ade (0.3%) in the preparation of lyxo-F-Ade that was used in these studies.

CONCLUSION

Although we were successful in designing *E. coli* PNP mutants that cleaved certain substrates much better than the wild-type enzyme, these compounds still had considerable toxicity that limited their usefulness. Our results indicated that the toxicity of methyl(talo)-MeP-R was due to its cleavage by bacterial enzymes to MeP. The enzyme responsible has not yet been identified, but it is not likely to be *E. coli* PNP since the MTD was only 5 times higher than that of MeP-dR and the rate of cleavage of methyl(talo)-MeP-R was 600-fold less than that of MeP-dR. Our results also indicated that the toxicity of lyxo-F-Ade and methyl(talo)-F-Ado was due to their cleavage by human MTAP to F-Ade. Further studies are underway to design new enzymes that can cleave nucleoside analogs that are not substrates for bacterial PNPs or human MTAP.

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