Synthesis, Biotransformation, and Pharmacokinetic Studies of 9-(β-D-Arabinofuranosyl)-6-azidopurine: A Prodrug for Ara-A Designed To Utilize the Azide Reduction Pathway[1]

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As a part of our efforts to design prodrugs for antiviral nucleosides, 9-(β-D-arabinofuranosyl)-6-azidopurine (6-AAP) was synthesized as a prodrug for ara-A that utilizes the azide reduction biotransformation pathway. 6-AAP was synthesized from ara-A *via* its 6-chloro analogue **4**. The bioconversion of the prodrug was investigated *in vitro* and *in vivo*, and the pharmacokinetic parameters were determined. For *in vitro* studies, 6-AAP was incubated in mouse serum and liver and brain homogenates. The half-lives of 6-AAP in serum and liver and brain homogenates were 3.73, 4.90, and 7.29 h, respectively. 6-AAP was metabolized primarily in the liver homogenate microsomal fraction by the reduction of the azido moiety to the amine, yielding ara-A. However, 6-AAP was found to be stable to adenosine deaminase in a separate *in vitro* study. The *in vivo* metabolism and disposition of ara-A and 6-AAP were conducted in mice. When 6-AAP was administered by either oral or intravenous route, the half-life of ara-A was 7−14 times higher than for ara-A administered intravenously. Ara-A could not be found in the brain after the intravenous administration of ara-A. However, after 6-AAP administration (by either oral or intravenous route), significant levels of ara-A were found in the brain. The results of this study demonstrate that 6-AAP is converted to ara-A, potentially increasing the half-life and the brain delivery of ara-A. Further studies to utilize the azide reduction approach on other clinically useful agents containing an amino group are in progress in our laboratories.

Introduction

Vidarabine, 9-(β-D-arabinofuranosyl)adenine (ara-A), was originally discovered as an antitumor agent,[2] and in later studies, it was shown to be active against herpes simplex virus types 1 and 2.[3,4] Ara-A is a licensed compound for the treatment of herpes simplex keratitis[5] and encephalitis.[6−8] It has also been considered for the treatment of genital and disseminated herpes infections,[9] cytomegalovirus encephalitis,[10] chronic hepatitis B virus (HBV) infection,[11,12] and acute non-lymphoid leukemia.[13] Ara-A may also be an alternative therapy for acyclovir-resistant herpes simplex virus, cytomegalovirus, and varicella-zoster virus infections.[14,15] However, the use of ara-A as a clinically effective agent is limited due to its rapid deamination to ara-H by adenosine deaminase (ADA) *in vivo*[16,17] as well as its poor solubility in water.

There were several attempts to prevent the rapid metabolism of ara-A,[18] including the coadministration of adenosine deaminase inhibitors such as deoxycoformycin[19−23] and N^6-benzoyladenosine.[24] The effects of ara-AMP and ara-A in combination with *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) were studied in mouse leukemia L1210/C2 cell culture, and the results were promising.[21] However, in the clinical trials with the combination of ara-A and deoxycoformycin, some patients developed toxicities.[25] Fludarabine is a 2-fluoro analogue of ara-A, which is resistant to deamination and proved to be effective in a number of luekemias.[26] However, in some patients reversible neurotoxicity and other neurological complications were observed.[27] Other approaches comprise the conjugation of ara-AMP to lactosaminated human serum albumin[28] and administration of ara-A in nanocapsules to improve the pharmacokinetic profiles.[29] 9-(β-D-Arabinofuranosyl)-6-(dimethylamino)purine (ara-DMAP), after intravenous administration in rats and monkeys, was rapidly converted to 9-(β-D-arabinofuranosyl)-6-(methylamino)purine (ara-MAP) and other purine metabolic end products.[30] However, less than 4% of the dose of ara-DMAP was found to be converted to ara-A, and the half-life of ara-A was 4 times longer.

As a part of our efforts to design prodrugs of antiviral agents with improved pharmacokinetic properties, we have recently synthesized 9-(2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-6-azidopurine (FAAddP) and 9-(2,3-dideoxy-2-fluoro-β-D-arabinofuranosyl)-N^6-methyladenine (FMAddA) as prodrugs of 2′-F-ara-ddI.[31] We have demonstrated that the 6-azido analogue FAAddP underwent reduction to 2′-F-ara-ddA by a human microsomal P-450 NADPH dependent system as well as in mice,[31] as the first example of the design of prodrugs taking advantage of the azide reduction pathway. As an extension of this approach, we have synthesized 9-(β-D-arabinofuranosyl)-6-azidopurine (6-AAP) as a prodrug of ara-A and wish to report here *in vitro* and *in vivo* pharmacokinetic studies.

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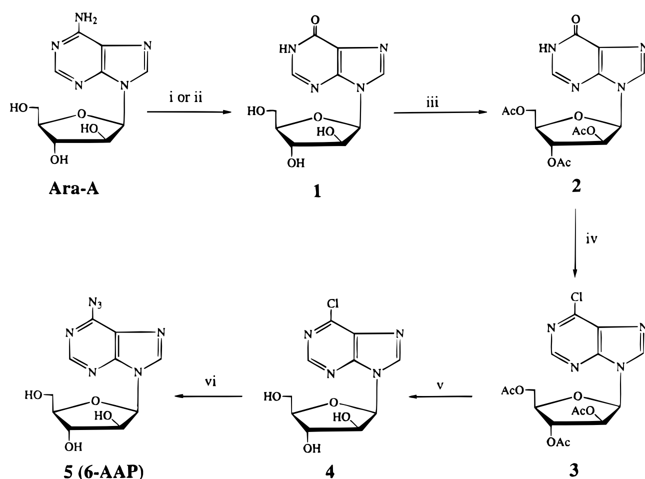
Scheme 1. Synthesis of 9-(*β*-D-Arabinofuranosyl)-6-azidopurine (6-AAP)[a]



[a] (i) $NaNO_2$, AcOH, 36 h; (ii) adenosine deaminase, water, 16 h; (iii) Ac_2O, pyridine, 0 °C, 16 h; (iv) SO_2Cl_2, CH_2Cl_2, DMF, reflux, 2 h; (v) NH_3, MeOH, 2 h; (vi) lithium azide, DMF, 2 days.

Results and Discussion

The target compound **5** (6-AAP) was synthesized from ara-A (Scheme 1). Ara-A was deaminated to 9-(*β*-D-arabinofuranosyl)hypoxanthine (**1**) using adenosine deaminase in >90% yield. This method was found superior in comparison to the deamination procedure with $NaNO_2$/AcOH. Compound **1** was peracetylated with acetic anhydride in pyridine and then converted to its 6-chloro derivative **3** under refluxing conditions with thionyl chloride (26% from **1**).[32] Compound **3** was deprotected to compound **4** by treatment with ammonia in methanol and subsequently treated with LiN_3 in DMF to obtain compound **5** (6-AAP) (38% from **3**).

The stability of 6-AAP at pH 2, 7, and 11 and toward adenosine deaminase hydrolysis was studied by UV spectroscopy. At pH 2 and 7, 6-AAP did not show any significant change over a period of 2.75 h at 287.5 nm (37.1 °C). However, at pH 11, 6-AAP immediately changed its UV absorption maximum from 287.5 to 222.5 nm. 6-AAP was not hydrolyzed by adenosine deaminase for up to 3 h in a separate *in vitro* study performed in phosphate buffer (pH 7.4) at 25 °C. These results show that unlike ara-A, 6-AAP is not a substrate for ADA.

Comparative *in vitro* studies were performed in mice liver homogenate by separately incubating 6-AAP and 6-AAP/coformycin to investigate the biotransformation of 6-AAP alone and in the presence of the ADA inhibitor coformycin (Table 1). The half-lives of 6-AAP in the absence and presence of coformycin were 4.90 and 5.98 h, respectively. Ara-A was detected in both cases, and the corresponding half-lives were 1.46 and 2.58 h, respectively. When ara-A alone was incubated in the mouse liver homogenate, its half-life was 0.04 h. Thus, the apparent half-life of ara-A generated from 6-AAP alone is 36 times greater than that of ara-A itself. Azido reduction assay utilizing the microsomal fraction of human liver homogenate also confirmed that 6-AAP was converted to ara-A as shown in mice liver homogenate studies. The biotransformation of 6-AAP to ara-A involves the cytochrome P-450 NADPH dependent system (Scheme 2). A similar reduction of the azido moiety of AZT to an amino function by the cytochrome P-450 system has been reported.[33,34] The stability and metabolism of 6-AAP were also studied in mice serum and brain homogenate (Table 1). The half-life of 6-AAP in mice serum and brain homogenate were 3.73 and 7.29 h, respectively. The decline of 6-AAP in serum was biphasic with a slow decline in the initial 1 h period ($T_{1/2}$ = 3.73 h) and then with a faster decline rate ($T_{1/2}$ = 1.41 h). No ara-A was detected in the serum *in vitro*, and thus the reason for the biphasic decline is not known at the present time. More rigorous biochemical studies are needed to be performed in order to explain this behavior of 6-AAP in serum.

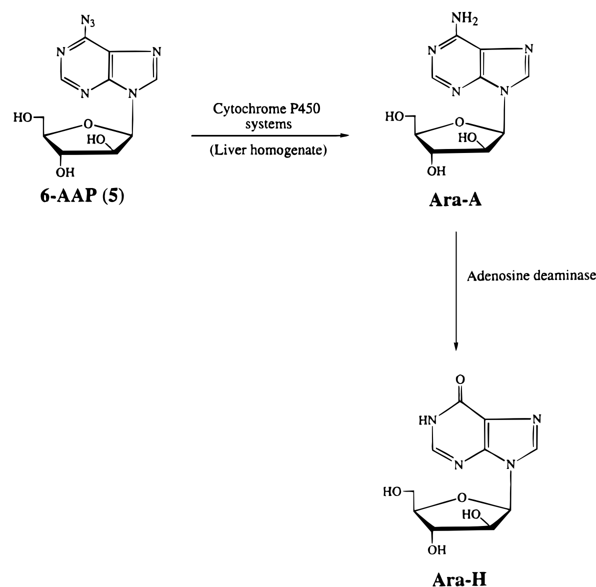
Following these interesting *in vitro* results, *in vivo* pharmacokinetic studies were performed in mice. Figure 1 shows the mean serum concentrations of 6-AAP versus time after intravenous and oral administration of 100 mg/kg 6-AAP. The corresponding pharmacokinetic parameters for 6-AAP are presented in Table 2. Maximum concentration of 6-AAP in serum was observed after 5 min of intravenous and after 60 min of oral dosing (Figure 1). Maximum concentrations of 6-AAP in serum after intravenous and oral dosing were 465 ± 167 and 7.8 ± 2.51 μg/mL, respectively. The terminal mean half-life values (0.55 and 0.58 h for intravenous and oral, respectively) were similar for both routes of administration. The area-under-curve (AUC)

Table 1. Parameters for the *in Vitro* Biotransformation of 6-AAP and Ara-A in Mice

compound	medium	analyte	K_{el} (h^{-1})	$T_{1/2}$ (h)
6-AAP[a]	liver homogenate	6-AAP	0.14	4.66
		ara-A	0.48	1.46
		ara-H	0.26	2.45
6-AAP and coformycin	liver homogenate	6-AAP	0.12	5.98
		ara-A	0.27	2.58
ara-A	liver homogenate	ara-A	16.87	0.04
		ara-H	0.31	2.24
6-AAP	serum[b]	6-AAP	0.25	3.73
6-AAP	brain homogenate	6-AAP	0.01	7.29

[a] Average of two studies, and the liver homogenate was prepared by addition of 1 or 1.5 weight equiv of water. [b] These values were calculated from 0 to 1 h after the incubation. The decline of 6-AAP concentration in serum was biphasic with a slow decline in the first 1 h and then with a faster decline ($T_{1/2}$ = 1.4 h).

Scheme 2. Biotransformation of 6-AAP to Ara-A in the Liver



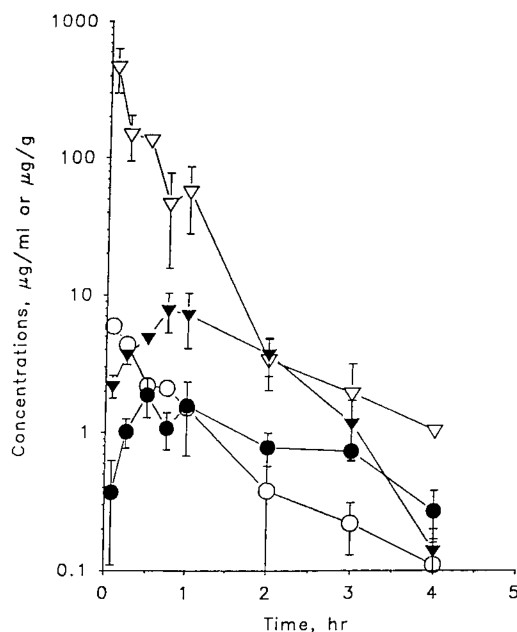


Figure 1. Mean ± SD concentrations of 6-AAP in serum (µg/mL) after intravenous (▽) and oral (▼) administration and in brain (µg/g) after intravenous (○) and oral (●) administration of 100 mg/kg 6-AAP to mice.

values for serum concentration versus time for 6-AAP were 201.1 ± 17.9 and 13.77 ± 1.4 mg·h/L, respectively, following intravenous and oral administration of 100 mg/kg 6-AAP. After intravenous administration of 20 mg/kg 6-AAP, the AUC value was 85.6 mg·h/L (data not shown), a 5-fold difference in the AUC values after intravenous dosing of 20 and 100 mg/kg 6-AAP, which indicates that the disposition of 6-AAP in mice followed linear kinetics in the dose interval between 20 and 100 mg/kg. There were no toxicities observed in the mice at either dose level, and the doses were well tolerated.

The concentrations of 6-AAP in brain versus time after intravenous and oral administrations are shown in Figure 1. After intravenous and oral administrations of 6-AAP, the maximum concentrations of 5.92 ± 0.7 and 1.87 ± 0.60 µg/g in the brain were observed at 5 and 30 min, respectively. The brain AUC values for 6-AAP were almost the same for both (intravenous and oral) routes of administration (4.41 ± 0.37 and 4.12 ± 0.37 mg·h/L, respectively) (Table 2). The serum AUC levels of 6-AAP were 201 ± 17.9 and 13.77 ± 1.40 mg·h/L after intravenous and oral administration of 6-AAP. In comparison, the brain AUC levels of 6-AAP are 2% and 33% of serum AUC levels after intravenous and oral administration of 6-AAP, respectively. These data suggest that there may be a saturable transport process of 6-AAP into the brain. Half-life of 6-AAP in the brain was approximately 2-fold greater after oral administration (1.29 h) than after intravenous administration (0.77 h). The relative brain exposure (r_e) value of 6-AAP was also greater after oral dosing (0.3) than after intravenous administration (0.02) of 6-AAP.

The serum concentrations of ara-A versus time after the administration of ara-A (intravenous) and 6-AAP (oral and intravenous) are shown in Figure 2. After the intravenous administration of ara-A, a significant fraction of the compound was rapidly metabolized, and its level declined from 18.5 ± 3.5 to 0.33 ± 0.25 µg/mL in 25 min. The AUC value was 3.95 ± 0.2 mg·h/L, and the half-life was 0.07 h. However, the pharmacokinetic curves for ara-A in serum after the intravenous administration of 6-AAP were different from those after the intravenous administration of ara-A. This curve revealed a "retard decline" of ara-A in serum with a significant increase in the half-life (0.89 h). Ara-A level was 0.28 ± 0.15 µg/mL after 3 h of the injection. The AUC value (6.84 ± 0.89 mg·h/mL) is 73% higher than that after the ara-A administration (3.95 ± 0.20 mg·h/mL) (Table 2). When 6-AAP (100 mg/kg) was administered orally, the serum AUC value of ara-A (1.15 ± 0.13 mg·h/L) was 29% of that after the intravenous administration of ara-A (3.95 ± 0.20 mg·h/L), and the half-life was 0.45 h.

The brain concentrations of ara-A after the administration of 6-AAP versus time are illustrated in Figure 3. Ara-A was not found in the brain after its intravenous administration in a dose of 100 mg/kg. There were no toxicities observed in mice at this level of ara-A. However, ara-A converted from 6-AAP exhibited a relatively constant mean concentration in the brain of 0.3−0.1 µg/g from 5 to 120 min after intravenous administration and from 5 to 240 min after oral administration of 6-AAP. The greater distribution in the brain was characterized by the increase in the brain AUC, r_e, and half-life values for ara-A after oral administration (1.55 ± 0.57 mg·h/L, 1.35, and 5.03 h, respectively) versus intravenous administration (0.35 ± 0.04 mg·h/L, 0.05, and 1.47 h, respectively) of 6-AAP (Table 2).

In summary, we have synthesized 9-(β-D-arabinofuranosyl)-6-azidopurine (6-AAP) as a prodrug of ara-A. In the *in vitro* studies in mice and human liver, 6-AAP was converted to ara-A by the cytochrome P-450 system. It was found that 6-AAP was not a substrate for adenosine deaminase. The *in vivo* pharmacokinetics in mice showed a significant improvement in the brain distribution and an increase in the half-lives of ara-A in the serum and the brain, after 6-AAP administration. It was also observed that the distribution of both 6-AAP and ara-A is independent of the route of administration of 6-AAP. Additional studies on 6-AAP as well as on utilizing the azido moiety in designing prodrugs for anticancer, antiviral, and other clinically useful agents containing an amino functionality with undesirable pharmacokinetic and toxicity profiles are in progress in our laboratories.

Experimental Section

Synthesis. Melting points were determined on a Mel-Temp II laboratory apparatus and are uncorrected. The ^{1}H NMR spectra were recorded on a JEOL FX 90 Q FT spectrometer with TMS as the internal standard; chemical shifts are reported in parts per million (δ), and the signals are quoted as s (singlet), d (doublet), t (triplet), or m (multiplet). UV spectra were recorded on a Beckman DU-650 spectrophotometer. TLC was performed on Uniplates (silica gel) purchased from Analtech Co. Elemental analysis was performed by Atlantic Microlab, Inc., Norcross, GA. Ara-A and adenosine deaminase (type II crude powder from calf intestinal mucosa, 1−5 units/mg of activity) were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade.

9-(β-D-Arabinofuranosyl)hypoxanthine (1). Method A: A solution of ara-A (500 mg, 1.87 mmol) in glacial acetic acid (8 mL) was treated with a solution of $NaNO_2$ (258 mg, 3.73 mmol) in 1 mL of water and stirred for 6 h. Three equal, additional portions of $NaNO_2$ (600 mg total, 8.4 mmol) were added over a 6 h period, and the stirring was continued. After 36 h, the solvent was evaporated *in vacuo*, and the residue was recrystallized from hot water (25 mL) to obtain pure **1** (339 mg, 67.6%): UV(MeOH) λ_{max} 249.0, 207.0 nm.

Table 2. Pharmacokinetic Parameters of Ara-A, 6-AAP, and Ara-A Released from 6-AAP after Dosing of 100 mg/kg Ara-A or 6-AAP

compound administered	route of administration	compound measured	tissue	AUC (mg·h/L)	$T_{1/2}$ (h)	r_e (brain)
ara-A	iv	ara-A	serum	3.95 ± 0.20	0.07	
			brain	ND[a]		
6-AAP	iv	6-AAP	serum	201.1 ± 17.9	0.55	
			brain	4.41 ± 0.37	0.77	0.02
		ara-A	serum	6.84 ± 0.89	0.89	
			brain	0.35 ± 0.04	1.47	0.05
6-AAP	oral	6-AAP	serum	13.77 ± 1.40	0.58	
			brain	4.12 ± 0.37	1.29	0.30
		ara-A	serum	1.15 ± 0.13	0.45	
			brain	1.55 ± 0.57	5.03	1.35

[a] ND, not detected.

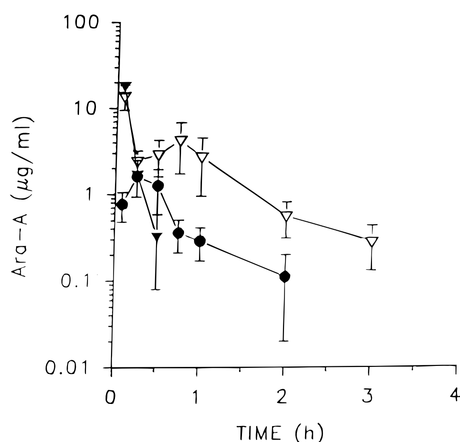


Figure 2. Mean ± SD serum concentrations of ara-A after intravenous administration of ara-A (▼) and after oral (●) and intravenous (▽) administration of 100 mg/kg 6-AAP to mice.

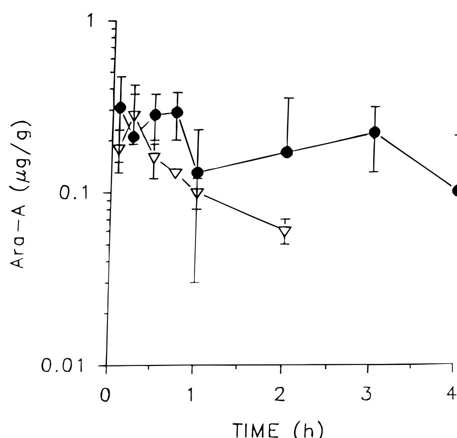


Figure 3. Mean ± SD brain concentrations of ara-A after oral (●) and intravenous (▽) administration of 100 mg/kg 6-AAP to mice.

Method B: A suspension of ara-A (500 g, 1.87 mmol) in distilled water (30 mL) was treated with adenosine deaminase (4 mg) and stirred for 16 h. The water was evaporated under reduced pressure, and the white residue was recrystallized from hot water (20 mL) to obtain compound **1** as a soft white solid (462 mg, 92%): UV (MeOH) λ_{max} 248.5, 205.5 nm.

9-(2,3,5-Tri-*O*-acetyl-*β*-D-arabinofuranosyl)hypoxanthine (2). Acetic anhydride (1 mL, 10.5 mmol) was added to a suspension of **1** (335 mg, 1.25 mmol) in dry pyridine (5 mL) at 0 °C and stirred for 16 h. The solvent was evaporated *in vacuo*, the residue was dissolved in 50 mL of methylene chloride, and the organic layer was washed with water (2 × 50 mL), saturated $NaHCO_3$ solution, and brine and dried (anhydrous sodium sulfate). The organic layer was concentrated *in vacuo* to obtain **2** as a brownish yellow solid (339 mg, crude yield 68%) which was used in the subsequent reaction without any further purification: UV (MeOH) λ_{max} 250.0, 206.0 nm.

6-Chloro-9-(2,3,5-tri-*O*-acetyl-*β*-D-arabinofuranosyl)purine (3). Crude compound **2** (130 mg) was dissolved in dry CH_2Cl_2 (10 mL) and heated to 55 °C. Dry DMF (1 mL) followed by a 2 M solution of $SOCl_2$ in CH_2Cl_2 (2.43 mL, 0.57 mmol) were added dropwise over a period of 45 min. The reaction mixture was gently refluxed for an additional 75 min. The reaction mixture was cooled to room temperature and diluted with CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ solution (2 × 50 mL) and brine (50 mL) and dried (anhydrous sodium sulfate). The organic phase was concentrated *in vacuo* and purified by preparative TLC (5% MeOH/$CHCl_3$) to obtain pure **3** (50 mg, 26% from **1**): UV (MeOH) λ_{max} 263.0, 212.5 nm.

9-(*β*-D-Arabinofuranosyl)-6-chloropurine (4). Compound **3** (200 mg, 0.5 mmol) was dissolved in saturated. NH_3/MeOH (5 mL) and stirred at room temperature for 2 h. The solvent was evaporated *in vacuo* to obtain crude **4** (170 mg) which was used for the subsequent reaction without any further purification: UV (MeOH) λ_{max} 263.0 nm.

9-(*β*-D-Arabinofuranosyl)-6-azidopurine (5). A solution of **4** (170 mg, 0.63 mmol) in DMF (5 mL) was treated with lithium azide (270 mg, 5.52 mmol) and stirred for 2 days at room temperature. The solvent was evaporated under reduced pressure at 40 °C, and the crude oil was recrystallized from MeOH to obtain pure **5** (67 mg, 38.4%): mp 185−190 °C dec; UV λ_{max} (water) pH 2, 205.0 (15 506), 287.0 nm (6496); pH 7, 208.5 (12 943), 287.5 nm (6033); pH 11, 222.5 nm (6730); ^1H NMR (DMSO-d_6) δ 3.66−3.90 (m, 3H, H-5′, H-4′), 4.16−4.33 (m, 2H, H-2′, H-3′), 5.14 (t, 1H, 5′-OH, exchangeable with D_2O), 6.50 (d, 1H, H-1′), 8.75 (s, 1H, H-8), 10.12 (s, 1H, H-2); IR (KBr) 2037, 1649. Anal. ($C_{10}H_{11}N_7O_4$·0.65CH_3OH) C, H, N.

Stability Studies of 6-AAP. A kinetic study at varying pHs (pH 2, 7, and 11 at 37.1 °C) was performed on a UV spectrophotometer to investigate the stability of 6-AAP. At pH 11, the UV absorption maximum for compound **5** shifted from 287.5 to 222.5 nm immediately. At pH 7, the compound did not show any significant change in UV absorption maxima over a period of 2.75 h at 287.5 nm indicating that it is stable at the neutral pH. At pH 2, the compound was stable.

Adenosine Deaminase Studies. 6-AAP (0.22 μM/mL) was incubated with adenosine deaminase (0.05 mg/mL) in phosphate buffer (pH 7.4) at 25.1 °C, and the change in the concentration was observed at 278.5 nm for 3 h.

Analysis. Concentrations of ara-A and 6-AAP in serum, brain (whole or homogenate), and liver homogenate were measured by high-performance liquid chromatography (HPLC). Chromatographic separations were carried out on a Millipore gradient system, which was equipped with a Model 486 tunable UV detector, two Model 510 pumps, a Model 717 plus autosampler, and Millennium 2010 Chromatography Manager software (Millipore Corp., Milford, MA). All the solvents were of HPLC grade. Chromatography was performed on an Altech Hypersil ODS C_{18} column (5 μm particle size, 4.6 × 150 mm; Altech Associates, Deerfield, IL). The mobile phase A was 0.5% acetonitrile in 2.5 mM KH_2PO_4 (pH 6.8), mobile phase B was 12% acetonitrile in 2.5 mM KH_2PO_4 (pH 6.8), mobile phase C was 2.5 mM KH_2PO_4 (pH 5.2), and mobile phase D was 23% methanol in 2.5 mM KH_2PO_4.

***In vitro* Metabolism Study.** Female NIH Swiss mice (Harland Sprague−Dawley, Indianapolis, IN) were sacrificed,

and serum and liver and brain tissues were collected before each experiment. Serum was collected from several animals. The brain and liver were washed in normal saline at 4 °C, wiped, and then weighed. Either 1 or 1.5 weight equiv of water was added to the tissue and homogenized using a homogenizer. The homogenate was divided into two halves: One portion was used as the blank and the other for incubation with either ara-A, 6-AAP or 6-AAP/coformycin in a water bath shaker at 37 °C. Initial concentrations of 6-AAP and ara-A were 100 μg/mL. Samples and blanks of volume 400 μL were collected at 0, 5, 15, and 30 min and at 1, 2, 3, 4, and 5 h.

To measure the analyte concentrations in serum or liver or brain homogenate, 400 μL of sample was mixed with 50 μL of internal standard (AzdU, 5 μg/mL) and 0.7 mL of acetonitrile. After centrifugation, the supernatant was decanted to another tube, treated with anhydrous Na_2SO_4, and then vortexed for 1 min and centrifuged again. The organic layer was separated and evaporated under nitrogen stream at room temperature. The residue was reconstituted in mobile phase A and filtered through a MPS-I micropartition system (3kDa membranes) (Amicon Inc., Beverly, MA) by centrifugation for 50 min at 2000 rpm to further clean up the samples; 100 μL of filtrate was injected for analysis.

For the HPLC analysis, during the first 10 min, the flow rate was changed linearly from 1.5 to 1.0 mL/min and continued at 1.0 mL/min until the end of assay (67 min). In the first 20 min, the mobile phase consisted of 95% A and 5% B; from 20 min, a linear gradient was run for 55 min to reach 5% A and 95% B. After each analysis, the column was equilibrated for 10 min to initial conditions. The λ_{max} was set at 249 nm for the first 15 min to observe ara-H, from 15 to 35 min at 261 nm to observe ara-A, from 35 to 45 min at 285 nm to observe 6-AAP, and subsequently changed to 261 nm to observe AzdU. The retention times for ara-H, ara-A, 6-AAP, and AzdU were 14.1, 33.8, 40.9, and 49.3 min, respectively.

Azido Reduction Study. The procedure for the analysis of azido-reducing activity was described previously.[33] Briefly, incubation mixtures contained either 1.5 mg of human liver fraction protein (homogenate or supernatant fractions following centrifugation) or 1.5 mg of microsomal protein, 5.0 mM $MgCl_2$, 6.0 mM NADPH, and 0.4 mg/mL 6-AAP in 0.1 M phosphate buffer saline at pH 7.4 (final volume of 0.2 mL). The reaction was initiated by adding NADPH and conducted at 37 °C for 60 min under nitrogen. Reactions were terminated by heating at 100 °C for 30 s, and the proteins were removed by centrifugation at 14000*g* for 6 min. Aliquots (100 μL) were then analyzed for nucleosides by HPLC. Control incubations were performed in the absence of protein.

Inhibition of Azido Reduction Assay. Assays were performed using 1.5 mg of microsomal protein as described above following either a 45 s exposure to carbon monoxide or a 5 min preincubation with 1 mM metyrapone prior to the addition of NADPH.

***In Vivo* Pharmacokinetics.** Female NIH Swiss mice (Harland Sprague−Dawley, Indianapolis, IN) weighing 24−28 g were used for the pharmacokinetic experiments. Mice were acclimatized in a 12 h light/12 h dark, constant temperature (20 °C) environment for 1 week before the experiments.

In a randomized study, animals were administered either 20 or 100 mg/kg 6-AAP or 100 mg/kg ara-A (intravenous). 6-AAP was also dosed orally (100 mg/kg po). At least three animals each were sacrificed at 0.08, 0.025, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 h after drug administration. Blood (serum) from the heart and whole-brain samples were collected. Serum samples were treated immediately, and brain samples were frozen at −20 °C until analysis. To determine the nucleoside concentrations in the serum, a known amount of serum sample, 50 μL of the internal standard (AzdU, 5 μg/mL), and 1.0 mL of acetonitrile as a protein precipitant were added to polypropylene microcentrifuge tubes (1.7 mL). Tubes were mixed and centrifuged at 9000 rpm for 10 min.

To measure the ara-A and 6-AAP in the whole brain, 50 μL of internal standard (AzdU, 10 μg/mL) and 300 μL of water were added to the weighed tissue samples (approximately 300 mg). After homogenization, 1.8 mL of acetonitrile was added to tissue homogenates, and samples were mixed and centrifuged at 9000 rpm for 10 min. The resulting supernatant from the serum or the brain was transferred to a clean tube and dried under a stream of nitrogen gas at 22 °C. The residue was reconstituted in 220 μL of mobile phase D, and after centrifugation at 12 000 rpm for 40 min, 100−150 μL was injected for the HPLC analysis. During the first 28 min, a linear gradient from 5% C and 95% D to 20% C and 80% D was run, and then during the next 20 min, a linear gradient was run to reach 65% C and 35% D at a flow rate of 1.5 mL/min. After each assay, the column was equilibrated to initial conditions for 7 min. The λ_{max} was set at 249 nm for the first 15 min to observe ara-H, from 15 to 30 min at 261 nm to observe ara-A, from 30 to 40 min at 285 nm to observe 6-azidoarabinosylpurine, and then changed to 261 nm to observe AzdU. The retention times for ara-H, ara-A, 6-AAP, and AzdU were 13.5, 27.8, 36.7, and 43.5 min, respectively.

Standard Curves. Standard curves were prepared for each type of sample by adding known amounts of ara-A and 6-AAP to the serum, brain, or liver and subjecting them to the extraction procedure as described above. The limits of quantitation of the ara-A and 6-AAP were 0.1 and 0.3 μg/mL, respectively. The percent recoveries of the compounds were 63% for ara-A and 55% for 6-AAP.

Data Analysis. Serum and tissue concentrations versus time data for 6-AAP and ara-A were analyzed by noncompartmental methods. The AUC versus time profiles from time zero to the last measured concentration were determined by the linear trapezoidal rule, and the AUC from the time of the last measured concentration to infinity was determined by dividing the last determined concentration by the least-squares elimination rate constant (λ_z). Half-life was calculated from 0.693/λ_z. The relative tissue exposure (r_e) of the compounds was calculated from AUC_{tissue}/AUC_{serum}. A variation of AUC was calculated according to previous procedures.[35]

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