Articles

In Vivo Biodistribution, Pharmacokinetic Parameters, and Brain Uptake of 5-Halo-6-methoxy(or ethoxy)-5,6-dihydro-3′-azido-3′-deoxythymidine Diastereomers as Potential Prodrugs of 3′-Azido-3′-deoxythymidine

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A new class of 5-halo-6-alkoxy-5,6-dihydro-3′-azido-3′-deoxythymidine diastereomers (5-X-6-OR-5,6-dihydro-AZTs; X = I, Br, Cl; R = Me, Et) were evaluated as potential anti-AIDS prodrugs of 3′-azido-3′-deoxythimidine (AZT). *In vivo* regeneration of AZT from these 5-X-6-OR-5,6-dihydro-AZTs was examined in Balb/c mice after intravenous tail vein injection. The (5*R*,6*R*)- and (5*S*,6*S*)-5-bromo(or iodo)-6-methoxy-5,6-dihydro derivatives of AZT (BMAZT, IMAZT) were rapidly converted to AZT, resulting in AZT plasma concentrations after a 144 μmol/kg dose similar to those after an equivalent dose (144 μg/kg, 38.5 mg/kg) of AZT, whereas AZT was not detectable by HPLC after the same dose of the chloro diastereomer (5*R*,6*R*)-CMAZT. The interaction of AZT and the 5-X-6-methoxy-5,6-dihydro-AZT diastereomers with the 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine equilibrative-sensitive nucleoside transporter in murine erythrocytes was also studied. The (5*R*,6*R*)- and (5*S*,6*S*)-5-X-6-OMe-5,6-dihydro-AZT diastereomers demonstrated a high affinity (K_i = 0.2−0.5 mM) for the transporter relative to AZT (K_i = 1.3 mM), with the exception of (5*S*,6*R*)-5-chloro-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (CMAZT) which has a K_i value larger than 1.5 mM. [2-^{14}C]-Labeled (5*R*,6*R*)- and (5*S*,6*S*)-5-bromo-6-methoxy(or ethoxy)-5,6-dihydro-3′-azido-3′-deoxythymidines were synthesized by the regiospecific addition of methyl hypobromite or ethyl hypobromite to the 5,6-olefinic bond of [2-^{14}C]-AZT in high radiochemical yield [(5*R*,6*R*)-BMAZT, 48%, and (5*S*,6*S*)-BMAZT, 33%; (5*R*,6*R*)-BEAZT, 61%, and (5*S*,6*S*)-BEAZT, 15%), high radiochemical purity (>98%), and high specific activity (56 mCi/mmol)]. The amounts of radioactivity in mouse brain after iv injection of [2-^{14}C]-labeled (5*R*,6*R*)-BMAZT, (5*S*,6*S*)-BMAZT, or (5*R*,6*R*)-BEAZT were 2−4 fold higher than that for [2-^{14}C]-AZT ($P < 0.05$). The radioactivity remaining in blood after dosing with these 5-bromo-6-alkoxy-5,6-dihydro-AZTs was up to 20-fold higher than after injection of [2-^{14}C]-AZT at longer time intervals after injection. The amounts of radioactivity present in femoral bone following injection of [2-^{14}C]-AZT, or these 5-bromo-6-alkoxy-5,6-dihydro-AZTs, were similar. Subcellular and regional distributions of [2-^{14}C]-labeled AZT, (5*R*,6*R*)-BMAZT, or (5*R*,6*R*)-BEAZT in mouse brain after jugular vein injection did not show preferential concentration in any particular subcellular fraction nor a marked preferential regional localization for either AZT or these 5,6-dihydro prodrugs of AZT.

3′-Azido-3′-deoxythymidine (AZT, zidovudine, Retrovir), the first drug approved for the treatment of human immunodeficiency virus (HIV), induces immunologic, virologic, and neurologic improvements in HIV-1-infected patients.[1] Thus, AZT therapy extends the life expectancy of individuals with acquired immunodeficiency syndrome (AIDS).[2] HIV can enter and replicate in the central nervous system (CNS)[3], causing adverse neurological dysfunctions. The most frequent dysfunction is AIDS-dementia complex, which includes motor, cognitive, and behavioral impairments that culminate in morbidity at advanced stages of the infection.[4−8] Although AZT, which inhibits HIV-encoded reverse transcriptase (RT) after conversion to AZT-5′-triphosphate by host cell kinases,[9,10] reduces mortality and morbidity in some AIDS patients, bone marrow toxicity resulting in anemia and leukopenia detract from its clinical utility.[11,12] Pharmacokinetic studies indicate that the half-life of AZT in plasma is approximately 1 h, which necessitates frequent doses to maintain effective therapeutic AZT levels.[13,14] Although AZT enters cerebrospinal fluid readily, its ability to cross the blood−brain barrier (BBB) to enter the brain is less than optimal.[14−16] Since the infected brain may serve as a sanctuary for the virus, from which the periphery may continuously be reinfected,[3] it is essential that the anti-HIV agent crosses the BBB readily to deliver an effective therapeutic concentration in brain without increasing toxicity.

A variety of prodrugs of AZT have been investigated to circumvent some of the limitations associated with AZT therapy such as inadequate delivery into brain, dose-related bone marrow toxicity, and rapid clearance from plasma. Previous studies involved esterification of the 5′-hydroxyl group of AZT,[17,18] utilization of

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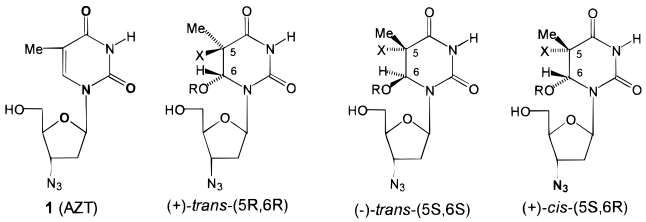
masked phosphate nucleotides,[19−21] attachment of a chemical delivery system,[22] and incorporation of another anti-HIV moiety.[23] Our approach[24−26] to overcoming the limitations associated with the efficacy of AZT (**1**)

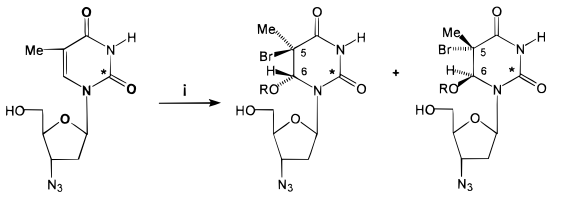


therapy is based on elaboration of the 5,6-olefinic bond of AZT to a 5-halo-6-alkoxy-5,6-dihydro analog, which increases lipophilicity significantly.[27] In an earlier study, the syntheses, antiviral activities, and *in vitro* biological stability of selected 5-halo-6-alkoxy-5,6-dihydro-3′-azido-3′-deoxythymidine diastereomers (**2**−**4**, 5-X-6-OR-5,6-dihydro-AZTs) were investigated as potential prodrugs to AZT to enhance the duration of action, lipophilicity, and cephalic delivery of AZT into the brain.[27] *In vitro* anti-HIV test data indicated that the substituents, and their configurations, at the the C-5 (X) and C-6 (alkoxy) positions of the 5,6-dihydro compounds **2**−**4** were determinants of anti-HIV activity. Previous *in vitro* evaluations[27] of selected 5-X-6-OR-dihydro-AZTs (**2**−**3**) indicated that these 5,6-dihydro analogs possess a number of desirable properties which enhance their potential as candidate prodrugs. These properties included an enhanced lipophilicity ($P = 3.3-18.8$) relative to AZT ($P = 1.29$) and stability to phosphorolysis by *Escherichia coli* thymidine phosphorylase. In addition, there was a correlation between their capacity to undergo *in vitro* regeneration to AZT and their anti-HIV potency. For example, the 5-iodo(or bromo)-6-methoxy-5,6-dihydro-AZTs, which undergo rapid conversion to AZT (I > Br) upon incubation with glutathione, mouse blood, or a soluble enzyme fraction of mouse liver, exhibited anti-HIV activity comparable to that of AZT (I ≥ Br). In contrast, the 5-chloro-6-methoxy-5,6-dihydro-AZTs, which did not undergo regeneration to AZT under these incubation conditions, exhibited a significantly decreased anti-HIV activity.[27] These beneficial properties of 5-X-6-OR-5,6-dihydro-AZTs (**2**−**3**) prompted us to investigate further their potential as anti-HIV prodrugs. We now report the ^{14}C-labeling, *in vivo* regeneration of AZT; plasma, brain and femoral bone biodistribution; pharmacokinetic parameters; and brain uptake data for selected 5-X-6-OR-5,6-dihydro-AZTs. In addition, the interaction (K_i, *zero-trans* influx inhibition values) of 5-halo-6-methoxy-5,6-dihydro-AZTs (**2**−**3**, X = I, Br, Cl) with the 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) facilitated equilibrative-sensitive (*es*) nucleoside transporter is also reported.

Chemistry

Reaction of [2-^{14}C]-AZT (**5**) with molecular bromine in methanol at 25 °C for 15 min and separation of the products by preparative HPLC afforded [2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-5-bromo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (**6a**, 48% chemical and radiochemical yield) and the [2-^{14}C]-(−)-*trans*-(5*S*,6*S*)-diastereomer (**7a**, 33% chemical and radiochemical yield) with a specific activity of 56 mCi/mmol and a radiochemical purity >98% (Scheme 1). A similar reaction of [2-^{14}C]-AZT with bromine in ethanol at 25 °C for 1.5 h and separation of the products by preparative HPLC gave [2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-5-bromo-6-ethoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (**6b**, 61% chemical and radiochemical yield) and the [2-^{14}C]-(−)-*trans*-(5*S*,6*S*)-diastereomer (**7b**, 15% chemical and radiochemical yield) with the same specific activity and radiochemical purity. The HPLC retentions times of the [2-^{14}C]-labeled products **6a**, **7a**, **6b**, and **7b** were identical to those of unlabeled authentic samples described previously.[27]

Scheme 1[a]



[a] Reagents: (i) Br_2, MeOH, 25 °C (products **6a**, **7a**); Br_2, EtOH, 25 °C (products **6b**, **7b**).

Results and Discussion

The objectives of this study involved additional *in vitro*[27] and *in vivo* evaluations of 5-halo-6-alkoxy-5,6-dihydro-3′-azido-3′-deoxythymidines, which were designed to be brain-targeted anti-HIV drugs and/or prodrugs to AZT. The chemotherapeutic value of nucleosides is dependent upon an understanding of the mechanism by which they exert their effect *in vivo*. Since activity is often dependent upon entry into intracellular metabolic pathways, an ability to cross the cell membrane is a requisite for effectiveness. Results from a recent study[28] suggest that AZT passes the cell monolayer mainly by passive diffusion, that an organic anion carrier which is inhibited by probenecid is involved in AZT transport from cerebral spinal fluid (CSF) to blood but not from blood to CSF, and that a nucleoside carrier system is not operative in AZT transport into and out of the CNS. Since phosphorylation of thymidine is competitive to AZT anabolism to its triphosphate, inhibition of thymidine transport into cells will result in an elevated intracellular AZT:thymidine ratio, which would favor AZT phosphorylation. This observation is consistent with the fact that the thymidine transport inhibitor dipyridamole potentiates the activity of AZT in monocytes/macrophages, which is due at least in part to suppression of both thymidine transport and its subsequent phosphorylation.[29,30] It was therefore of interest to determine the effect which reduction of the 5,6-olefinic bond of AZT and introduction of C-5 halo and C-6 alkoxy substituents have on transportability.

Thymidine Influx Inhibition Constants (K_i). The effect of (+)-*trans*-(5*R*,6*R*)- and (+)-*cis*-(5*S*,6*R*)-5-chloro-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (CMAZT), (+)-*trans*-(5*R*,6*R*)- and (−)-*trans*-(5*S*,6*S*)-5-bromo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (BMAZT), (+)-*trans*-(5*R*,6*R*)- and (−)-*trans*-(5*S*,6*S*)-

Table 1. Thymidine Influx Inhibition Constants (K_i) for 5-Halo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidines, AZT, and 2′-Deoxyuridine in Fresh Murine Erythrocytes

compound	configuration	K_i (mM)[a]	P[b]
(+)-*trans*-CMAZT	5*R*,6*R*	0.36 ± 0.01	7.6
(+)-*cis*-CMAZT	5*S*,6*R*	>1.5	3.3
(+)-*trans*-BMAZT	5*R*,6*R*	0.45 ± 0.07	13.2
(−)-*trans*-BMAZT	5*S*,6*S*	0.50 ± 0.04	16.7
(+)-*trans*-IMAZT	5*R*,6*R*	0.35 ± 0.03	10.6
(−)-*trans*-IMAZT	5*S*,6*S*	0.20 ± 0.03	18.8
AZT		1.33 ± 0.04	1.3
DU[c]		0.125 ± 0.021	

[a] Data are expressed as the mean ± SD, $n = 3$. [b] P = 1-octanol/water partition coefficient values taken from ref 27. [c] DU = 2′-deoxyuridine; the K_i value is taken from ref 35.

5-iodo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (IMAZT), AZT, and the physiological nucleoside 2′-deoxyuridine (DU) on entry of thymidine into fresh murine erythrocytes was measured by determination of their thymidine transport inhibition values (K_i) according to the method previously described.[31,32] The K_i represents the relative affinity of a test compound for the external binding sites of the NBMPR equilibrative-sensitive (*es*) transporter, with a larger K_i indicating a lower test compound affinity for external binding sites of the nucleoside transporter. All of the (+)-*trans*-(5*R*,6*R*)- and (−)-*trans*-(5*S*,6*S*)-5-halo-6-methoxy-5,6-dihydro analogs (CMAZT, BMAZT, IMAZT) of AZT exhibited a higher affinity for the NBMPR transporter (K_i = 0.2−0.5 mM range) than AZT (K_i = 1.33 mM), but a lower affinity than that of the natural nucleoside 2′-deoxyuridine (K_i = 0.125 mM), as summarized in Table 1. In contrast, (+)-*cis*-(5*S*,6*R*)-CMAZT had a K_i of >1.5 mM. This difference demonstrates the importance of configuration (stereoselectivity)[33] for inhibiting the transporter. A molecular model of the permeant binding site of the *es* transporter derived from three-dimensional structures, physicochemical properties, and quantitative structure−activity data for various nucleoside permeants predicts that the permeant-binding site of the *es* transporter is sensitive to the size and hydrophobicity of the heterocyclic base moiety, prefers nucleosides in the *anti* conformation, and has residues that hydrogen-bond to the 5′-OH of the sugar moiety.[34] There are two possible interpretations for the increased affinity of the 5-halo-6-methoxy-5,6-dihydro-AZTs for the NBMPR transporter. One possibility is that these 5,6-dihydro analogs are transported by the NBMPR transporter. The second possibility is that they bind to the transporter but are not transported, and the smaller K_i value is due to enhanced hydrophobic binding of the 5,6-dihydro compound to the transporter binding sites. The observation that compounds which are weak transport inhibitors ($K_i \geq 1.33$ mM) were less lipophlic (P = 1.3−3.3), whereas the more potent inhibitors (K_i = 0.2−0.5 mM) were more lipophilic (P = 7.6−18.8) provides support for the latter possibility (see data in Table 1). Irrespective of whether these 5-halo-6-methoxy-5,6-dihydro-AZTs are or are not transported by the NBMPR sensitive facilitated transport system, the (+)- and (−)-*trans*-analogs do inhibit the influx of thymidine more effectively than AZT. Although these 5-halo-6-methoxy-5,6-dihydro prodrugs are more potent inhibitors of thymidine uptake into erythrocytes than AZT, their K_i values (0.2−0.5 mM range) are much larger than the therapeutic concentration of AZT (5−20 μM range). It is therefore unlikely that there would be a significant effect on the AZT:thymidine ratio in tissues at relevant concentrations.

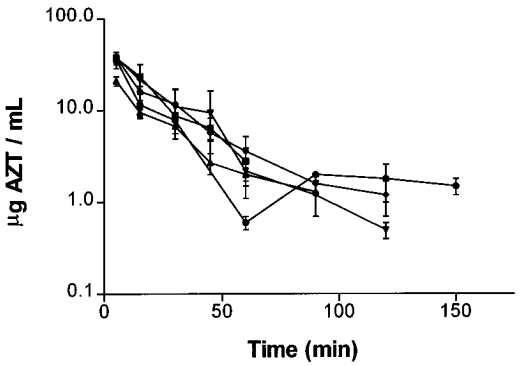


Figure 1. Concentration (μg/mL) of regenerated AZT in plasma samples after iv injection (144 μmol/kg dose) of AZT (■), (5*R*,6*R*)-BMAZT (▲), (5*S*,6*S*)-BMAZT (▼), (5*R*,6*R*)-IMAZT (◆), and (5*S*,6*S*)-IMAZT (●) into Balb/c mice. The data are expressed as the mean ± SD (n= 3). The AZT detection limit by HPLC was about 0.5 μg/mL.

Table 2. Pharmacokinetic Parameters for AZT After Intravenous Administration (144 μmol/kg) of AZT and 5-Bromo(or iodo)-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine Diastereomers into Balb/c Mice in Plasma Samples

compound administered	AUC[a,b] (mg·min·mL^{-1})	$t_{1/2}$[a,c] (min)
AZT	707.1	23.5
(5*R*,6*R*)-BMAZT	404.6	41.8
(5*S*,6*S*)-BMAZT	807.4	31.1
(5*R*,6*R*)-IMAZT	748.6	33.6
(5*S*,6*S*)-IMAZT	589.3	148.6

[a] Mean value for AZT after injection of AZT or the 5,6-dihydro prodrugs (n = 3). [b] Area under the time−concentration curve (0 → last sample). [c] Elimination half-life calculated using a one-compartment model.

Pharmacokinetic Parameters for AZT after *in Vivo* Regeneration of AZT from 5-Halo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidines. The value of a prodrug is primarily dependent upon its conversion to the parent drug since the prodrug is usually inactive. A sustained release of the parent drug from the prodrug is particularly important for drugs having narrow therapeutic indices. It is known that AZT exhibits dose-related bone marrow toxicity, and to reduce this toxicity, lower doses of AZT have been recommended.[36] However, this may result in lower brain levels, thereby decreasing the therapeutic efficacy of AZT for treating cerebral infections.[37] To obtain a balance between an adequate concentration for efficacy and a lower concentration for reduced toxicity, a lipophilic prodrug having a longer residence time in the body is needed. The regeneration of AZT from 5-halo-6-methoxy-5,6-dihydro-AZTs was investigated after intravenous (iv) injection (144 μmol/kg dose) into Balb/c mice and compared with the AZT concentrations after an equivalent (144 μmol/kg, 38.5 mg/kg) dose of AZT. The concentration of AZT in plasma was determined by HPLC analysis using 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-iodouracil (FIAU) as an internal standard. The recoveries of AZT and FIAU from Sep-Pak cartridges were 88 ± 5% and 84 ± 4%, respectively. The plasma levels (μg/mL) of AZT (see Figure 1) and pharmacokinetic parameters (AUC, $t_{1/2}$) for regenerated AZT (see Table 2) after iv doses of AZT, (+)-*trans*-(5*R*,6*R*)-BMAZT, (−)-*trans*-(5*S*,6*S*)-BMAZT, (+)-*trans*-(5*R*,6*R*)-IMAZT, or (−)-*trans*-(5*S*,6*S*)-IMAZT were determined. These results indicate that the

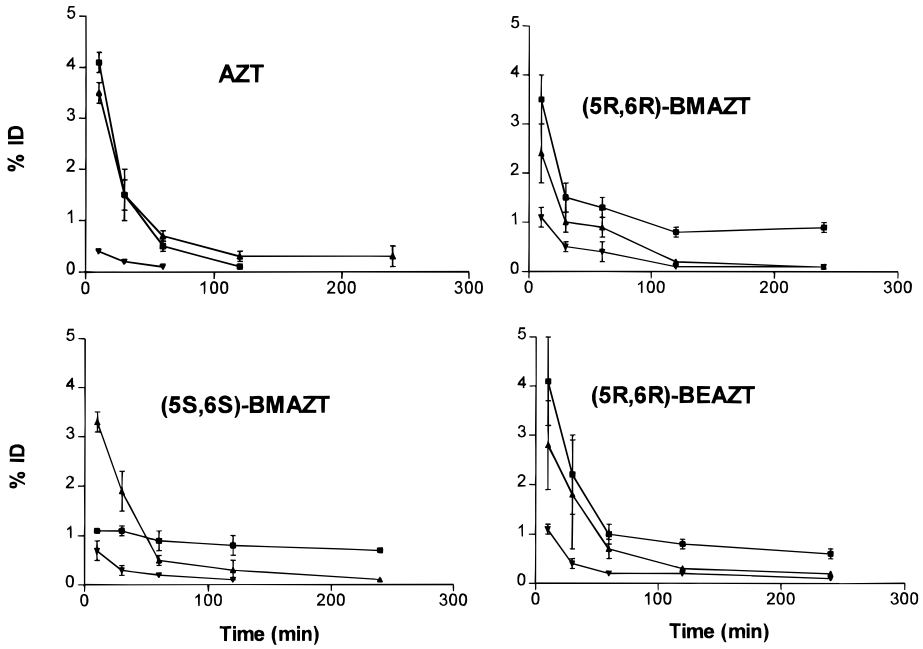


Figure 2. Distribution of analyte radioactivity in blood (■), brain (▼), and femoral bone (▲) samples from Balb/c mice after iv injection of [2-^{14}C]-labeled AZT, (5*S*,6*S*)- BMAZT, (5*R*,6*R*)-BMAZT, and (5*R*,6*R*)-BEAZT (2 μCi/mouse, 35.5 pmol dose). Data are expressed as the mean ± SD ($n = 3$) of the percentage of the injected dose per milliliter of blood or gram of brain or femoral bone. The analyte species could be administered compound, AZT, and/or a metabolite.

BMAZT and IMAZT diastereomers are all rapidly converted to AZT *in vivo*, thereby providing qualitatively similar plasma levels of AZT to that observed after AZT administration. In contrast, the extent of AZT regeneration from the same dose (144 μmol/kg) to mice of the chloro diastereomer (+)-*trans*-(5*R*,6*R*)-CMAZT, which was neglible, was below the HPLC detection limit of about 0.5 μg/mL. However, after administration of a 240 μmol/kg iv dose of the latter chloro diastereomer, the blood concentrations (μg/mL, average of two experiments) of AZT and CMAZT, respectively, were 2.5 and 28.8, 2.5 and 12.5, 1.3 and 16.3, and <1 and 1.1 μg/mL at 15, 20, 60, and 90 min postinjection.

The AUC for AZT after iv administration of (5*R*,6*R*)- and (5*S*,6*S*)-IMAZT was lower than that for AZT. In contrast, (5*S*,6*S*)-BMAZT and (5*R*,6*R*)-IMAZT exhibited slightly elevated AUC values for AZT, relative to that observed after administration of AZT (see Table 2). The elimination of AZT and all 5,6-dihydro prodrugs investigated, except for (5*S*,6*S*)-IMAZT, from plasma followed a one-compartment kinetic model. However, the concentration of AZT in plasma after iv administration of (5*S*,6*S*)-IMAZT showed a second C_{max}, which resulted in a longer $t_{1/2}$ of 149 min for AZT in plasma.

A previous study suggested that the *in vitro* regeneration of uracil nucleosides from the 5,6-dihydro derivatives is a thiol-mediated process and that regeneration of the 5,6-olefinic bond upon incubation with glutathione in phosphate buffer (pH 7.4) at 37 °C is dependent on the relative glutathione concentration and the nature of the halogen atom (I, Br, Cl) at the C-5 position.[27] Since it is known that glutathione levels in HIV-infected subjects are about one-third those of uninfected individuals,[38] the regeneration of AZT from these 5-halo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidines in infected subjects is difficult to predict.

On the basis of the proposed regeneration mechanisms[27] and previous reports of dehalogenation of 5,6-dihydrouracil nucleosides,[24−26,39,40] it is likely that the alkoxy moiety at the C-6 position also has an impact on the rate of AZT regeneration. Therefore, it may be possible to design a 5-halo-6-alkoxy-5,6-dihydro prodrug of AZT with the desired rate of conversion to AZT by selecting an appropriate combination of C-5 halo and C-6 alkoxy substituents.

Biodistribution of Analyte Radioactivity in Blood, Brain, and Femoral Bone after Administration of [2-^{14}C]-Labeled AZT, (+)-*trans*-(5*R*,6*R*)- and (−)-*trans*-(5*S*,6*S*)-BMAZT, and (+)-*trans*-(5*R*,6*R*)-BEAZT to Balb/c Mice. The class of 5-halo-6-alkoxy-5,6-dihydro-3′-azido-3′-deoxythymidines being investigated was designed to provide prodrugs of AZT with an enhanced lipophilicity, duration of action, and delivery into the CNS (brain). Accordingly, the biodistributions of the selected title compounds following iv injection were determined. The levels of analyte radioactivity (the analyte species could be the administered compound, AZT and/or a metabolite) in blood, brain, and femoral bone after injection of 2 μCi (35.5 pmol dose) were determined using a tissue combustion/liquid scintillation counting method. The results, expressed as the percentage of administered radioactivity per gram or milliliter of tissue [% injected dose (ID)/g or mL] are shown in Figure 2 for AZT, (+)-*trans*-(5*R*,6*R*)-BMAZT, (−)-*trans*-(5*S*,6*S*)-BMAZT, and (+)-*trans*-(5*R*,6*R*)-BEAZT. The analyte radioactivity of all four [2-^{14}C]-labeled compounds was distributed to all tissues examined (including heart, liver, lung, spleen, kidney, bladder; data not shown) after iv injection into Balb/c mice. Moreover, the analyte radioactivity levels in most tissues were higher after injection of the [2-^{14}C]-5-bromo-6-alkoxy-AZTs than after [2-^{14}C]-AZT dosing, especially at longer time intervals after dosing.

The analyte radioactivity level in blood after (5*R*,6*R*)-BMAZT and (5*R*,6*R*)-BEAZT administration was significantly higher than after injection of AZT, particularly at time periods of >100 min. In this study, the

Table 3. Distribution of Radioactivity and Quantity of Analyte (pmol/mg of protein) in Subcellular Fractions of Mouse Brain (pooled samples from five mice) after Jugular Vein Injection of [2-^{14}C]-AZT, [2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-BMAZT, and [2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-BEAZT (2 μCi, 35.5 pmol dose)

	% protein distribution (A)[a]			% radioactivity distribution (B)[b]			pmol of analyte/mg of protein (C)[c]			relative specific concentration (RSC = B/A)		
	AZT	BMAZT	BEAZT	AZT	BMAZT	BEAZT	AZT	BMAZT	BEAZT	AZT	BMAZT	BEAZT
P_1[d]	15.9	13.3	11.9	14.6	4.6	5.2	1.12	2.08	3.96	0.92	0.34	0.44
P_2[d]	51.7	53.1	43.0	7.6	5.6	7.7	0.28	0.65	1.62	0.15	0.11	0.18
P_3[d]	9.6	8.7	17.6	3.4	1.4	2.4	0.48	0.98	1.21	0.35	0.16	0.14
S_3[d]	22.8	24.9	27.5	74.5	88.4	84.7	10.16	21.80	27.79	3.30	3.55	3.10

[a] Percentage of total protein recovered. [b] Percentage of total radioactivity recovered. [c] Quantity of analyte in pmol/mg of protein. The analyte species could be the compound administered, AZT, and/or a metabolite. [d] The brain subcellular fraction P_1 contains nuclei and cell debris; P_2 contains myelin fragments, synaptosomes (pinched-nerve endings), and mitochondria; P_3 is the microsomal fraction; and S_3 is the soluble fraction.

AUCs, calculated by a trapazoidal method for fixed intervals [$AUC_{10-360min}$ (% ID·min/g)] were substantially higher for (5*R*,6*R*)-BMAZT and (5*R*,6*R*)-BEAZT (346 and 322% ID·min/g) than for AZT (116% ID·min/g). The prolonged blood levels of these prodrugs of AZT may therefore provide an improved therapeutic efficacy.

The analyte radioactivity levels in brain following iv injection of (5*R*,6*R*)-BMAZT and (5*R*,6*R*)-BEAZT were also up to 3-fold higher than for AZT, and the $AUC_{10-240min}$ in brain of these two 5,6-dihydro analogs (55.7 and 51.5% ID·min/g, respectively) was larger than that for AZT (17.4% ID·min/g). Neurological disorders, which contribute to the morbidity in advanced stages of HIV infection, are common in AIDS patients. Although AZT therapy improves CNS dysfunctions, the efficacy of AZT is less than desired due to its low ability to penetrate through the BBB.[16] The 5-bromo-6-alkoxy-5,6-dihydro-AZTs studied, which enter the brain more readily and provide drug concentrations up to 3-fold higher than does an equimolar dose of AZT, may therefore be more efficaceous anti-HIV drugs. These 5-bromo-6-alkoxy-5,6-dihydro-AZTs are expected to undergo regeneration to AZT in the brain since a related compound, (5*R*,6*R*)-5-bromo-5-ethyl-6-ethoxy-5,6-dihydro-2′-deoxyuridine, underwent conversion to 5-ethyl-2′-deoxyuridine upon incubation with a rat brain homogenate for 2 h (16%), relative to that in rat plasma (8%) and rat whole blood (53%).[41] The level of analyte radioactivity in femoral bone did not differ significantly upon injection of either AZT or the AZT prodrugs investigated (see Figure 2).

It must be recognized that only total analyte radioactivity was measured in these experiments and that the chemical species associated with the radioactivity were not characterized. However, it was reported[27] previously that (5*R*,6*R*)-BMAZT and (5*R*,6*R*)-BEAZT undergo 80% and 100% conversion to AZT upon *in* vitro incubation with glutathione at 37 °C for 30 min in phosphate buffer (pH 7.4), respectively. Incubation of (5*R*,6*R*)-BMAZT with mouse blood for 10 min at 37 °C resulted in a 59% conversion to AZT. These results, and previous metabolic studies of AZT,[42,43] suggest that the radioactive chemical species, which are putative metabolites of BMAZT or BEAZT, include AZT, AZT-MP (3′-azido-3′-deoxythymidine-5′-monophosphate), AZT-DP (3′-azido-3′-deoxythymidine-5′-diphosphate), AZT-TP (3′-azido-3′-deoxythymidine-5′-triphosphate), AMT (3′-amino-3′-deoxythymidine), GAMT (5′-O-glucuronide-3′-amino-3′-deoxythymidine), and GAZT (5′-O-glucuronide-3′-azido-3′-deoxythymidine).

Brain Subcellular and Regional Distributions of Analyte Radioactivity after Administration of [2-^{14}C]-Labeled AZT, (+)-*trans*-(5*R*,6*R*)-BMAZT, and (+)-*trans*-(5*R*,6*R*)-BEAZT to Balb/c Mice. Since increased levels of analyte radioactivity in brain were observed after doses of [2-^{14}C]-labeled (+)-*trans*-(5*R*,6*R*)-BMAZT, (−)-*trans*-(5*S*,6*S*)-BMAZT, and (+)-*trans*-(5*R*,6*R*)-BEAZT, the subcellular and regional distributions of analyte radioactivity in brain after jugular vein injection of the title compounds were determined. The amounts of analyte radioactivity in brain subcellular fractions P_1 (contains nuclei and cell debris), P_2 [contains myelin fragments, synaptosomes (pinched-nerve endings), and mitochondria], P_3 (microsomal fraction), and S_3 (soluble fraction) and in different regions including cerebellum, pons−medulla, hypothalamus, hippocampus, striatum, cortex, and the remainder of the brain are presented in Tables 3 and 4. The highest level of analyte radioactivity after dosing with [2-^{14}C]-labeled AZT, BMAZT, and BEAZT was present in the subcellular fraction S_3 (see Table 3). Although levels of analyte radioactivity tended to be slightly higher in hypothalmus, there was no evidence of marked selective accumulation of the radioactive species in any brain region (see Table 4).

Table 4. Distribution of Analyte (pmol/mg) in Mouse Brain Regions after Jugular Vein Injection of [2-^{14}C]-AZT, [2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-BMAZT, and [2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-BEAZT (2 μCi/mouse, 35.5 pmol dose)

	analyte concentration (pmol/mg) from pooled samples[a]		
	[2-^{14}C]-AZT[b]	[2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-BMAZT[c]	[2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-BEAZT[c]
cerebellum	0.14	0.72	0.66
pons & medulla	0.15	0.66	0.62
hypothalamus	0.21	1.32	1.10
hippocampus	0.14	0.71	1.10
striatum	0.14	0.88	0.85
cortex	0.12	0.71	0.66
rest of brain	0.13	0.71	0.83

[a] The analyte species could be the administered compound, AZT, and/or a metabolite. [b] Samples were pooled from four individual mice. [c] Samples were pooled from two individual mice.

Summary

A new class of 5-halo-6-alkoxy-5,6-dihydro-3′-azido-3′-deoxythymidines has been evaluated as prodrugs of AZT. With the exception of (+)-*cis*-(5*S*,6*R*)-5-chloro-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine, these 5-halo-6-alkoxy-5,6-dihydro analogs of AZT are better inhibitors than AZT of thymidine transport by the murine erythrocyte equilibrative-sensitive NBMPR nucleoside transport system. The 5-bromo(or iodo)-6-methoxy-5,6-dihydro-AZT prodrugs (144 μmol/kg iv

dose) are converted to AZT *in vivo* to give AZT concentrations in blood similar to that of an equivalent dose of AZT, whereas regeneration of AZT from an identical dose of the 5-chloro analog was neglible at the same dose. The 3-fold increase in radioactivity uptake by brain, compared to an equivalent dose of AZT, is attributed to their higher lipophilicity. Uptake in femoral bone was not significantly different after administration of [2-^{14}C]-labeled AZT or the 5,6-dihydro prodrugs investigated. The increased brain uptake was not associated with any preferential binding to a particulate subcellular fraction or to preferential regional localization compared to AZT. It is not known whether these 5,6-dihydro prodrugs will cause the myopathy induced by AZT, which may be due to inhibition of mitochondrial DNA synthesis.[44] The 5-bromo(or iodo)-6-alkoxy-5,6-dihydro prodrugs described, which are equipotent to AZT, could serve as useful lead compounds for the development of a prodrug to AZT on the basis of their enhanced brain uptake relative to AZT.

Experimental Section

All solvents and reagents used were of reagent quality. Methanol and ethanol were dried using standard methods and distilled just prior to use. HPLC separations of the products in the ^{14}C-labeling were performed using a Waters HPLC system comprised of Model 510 and M-45 solvent pumps, Model 860 gradient controller, Model U6K injector, and Model 480LC variable wavelength ultraviolet detector set at 230 nm. A Whatman Partisil M9 10/25 ODS reverse phase column was utilized in the analyses. Analytical HPLC studies were performed using similar HPLC systems having either a Hewlett Packard 79994A workstation and 1040A photodiode detector or a Waters 486 variable wavelength ultraviolet detector. TLC analyses were performed on Whatman MK6F silica gel microslides. The identity of each compound (diastereomer) was determined by comparison of their TLC R_f values and reverse phase HPLC retention times under various chromatographic conditions with unlabeled authentic samples previously characterized.[27] Radioactivity was determined by liquid scintillation counting using Aquasol fluor (New England Nuclear) with a Beckman LS9000 liquid scintillation counter. [*methyl*-^{3}H]-Thymidine was purchased from Amersham and NBMPR was obtained from the Aldrich Chemical Co. The unlabeled 5-halo-6-alkoxy-5,6-dihydro-3′-azido-3′-deoxythymidine diastereomers employed were prepared using methodologies previously reported.[27] [2-^{14}C]-3′-Azido-3′-deoxythymidine (specific activity 56 mCi/mmol) was purchased from Moravek.

[2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-5-Bromo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (6a) and [2-^{14}C]-(−)-*trans*-(5*S*,6*S*)-5-Bromo-6-methoxy-3′-azido-3′-deoxythymidine (7a). A solution of [2-^{14}C]-AZT (500 μCi, specific activity 56 mCi/mmol) in dry methanol (60 μL) was added to a solution of bromine (3 mg, 0.02 mmol) in dry methanol (90 μL), and the reaction was allowed to proceed at 25 °C for 15 min with stirring. Micro-TLC analysis indicated the reaction was complete. The reaction mixture was purified by preparative HPLC (6 injections) using water:acetonitrile (80:20, v/v) as eluant with a flow rate of 2 mL/min and detection at 230 nm to yield **6a** (184 μCi, 48% chemical and radiochemical yield, >98% radiochemical purity, specific activity = 56 mCi/mmol, retention time = 30.44 min) and **7a** (127 μCi, 33% chemical and radiochemical yield, >98% radiochemical putity, specific activity = 56 mCi/mmol, retention time = 34.55 min).

[2-^{14}C]-(+)-*trans*-(5*R*,6*R*)-5-Bromo-6-ethoxy-5,6-dihydro-3′-azido-3′-deoxythymidine (6b) and [2-^{14}C]-(−)-*trans*-(5*S*,6*S*)-5-Bromo-6-ethoxy-3′-azido-3′-deoxythymidine (7b). [2-^{14}C]-AZT (250 μCi, specific activity = 56 mCi/mmol) in dry ethanol (60 μL) was added to a solution of bromine (3 mg, 0.02 mmol) in dry ethanol (90 μL) and the reaction mixture was stirred at 25 °C for 1.5 h. Micro-TLC analysis at this time indicated that the reaction was complete. Purification of the reaction mixture by preparative HPLC (six injections) using water:acetonitrile (70:30, v/v) as eluant with a flow rate of 2 mL/min and detection at 230 nm afforded **6b** (153 μCi, 61% chemical and radiochemical yield, >98% radiochemical purity, specific activity = 56 mCi/mmol, retention time = 19.82 min) and **7b** (38 μCi, 15% chemical and radiochemical yield, 98% radiochemical purity, specific activity = 56 mCi/mmol, retention time = 22.17 min).

Thymidine Influx Inhibition Assay. The interaction of AZT, (5*R*,6*R*)- and (5*S*,6*S*)-IMAZT, (5*R*,6*R*)- and (5*S*,6*S*)-BMAZT, and (5*R*,6*R*)- and (5*S*,6*R*)-CMAZT with the NBMPR equilibrative-sensitive nucleoside transporter was examined in mouse erythrocytes using a method previously described in the literature.[31,32] Briefly, erythrocytes obtained from $B_6D_2F_1$ mouse blood were suspended at a 10% hematocrit in HEPES-buffered saline (HBS). The *zero-trans* influx was initiated by the rapid addition of HBS (200 μL) containing [*methyl*-^{3}H]-thymidine (40−60 Ci/mmol, 1 mCi/mL) solutions at four concentrations (0.1−1.0 mM) alone and together with four concentrations (0.1−2.5 mM) of test compound, to the erythrocyte suspension (200 μL). After 3 s, the influx was terminated by adding NBMPR to give a final concentration of 10 μM. Cells were pelleted by centrifugation at 12 800 rpm for 1 min (Eppendorf 5412 microcentrifuge) and washed once with NBMPR solution (1 mL). Cell pellets were extracted with ice-chilled 5% perchloric acid (0.5 mL). After at least 30 min of cooling at ice temperature, the mixture was subjected to centrifugation, and two aliquots (150 μL) of the supernatant were mixed with ScintiVerse II (15 mL) and counted. The *zero-time* value was obtained by reversing the order in which NBMPR was added to the cells prior to the addition of the permeant solution. This value was subtracted from radioactivity values in all influx samples. The experiment was conducted in duplicate. The inhibition constant K_i was estimated by plotting 1/v against i (v is the velocity of uptake, nmol of [^{3}H]-thymidine/10^{10} cells/s; *i* is the inhibitor concentration) according to the method of Dixon,[45] and the lines were fitted by the visual best fit and intersected at a point, for which $i = -K_i$.

The *in Vivo* Regeneration of AZT from 5-Halo-6-methoxy-5,6-dihydro-3′-azido-3′-deoxythymidines in Mice. The *in vivo* regeneration of AZT from (5*R*,6*R*)- and (5*S*,6*S*)-IMAZT, (5*R*,6*R*)- and (5*S*,6*S*)-BMAZT, and (5*S*,6*R*)- and (5*R*,6*R*)-CMAZT was investigated in Balb/c mice. The test compound dissolved in 10% DMSO/water (3.6 μmol/0.1 mL) was injected via the tail vein at a dose of 144 μmol/kg. The mice were sacrificed at specific times by asphyxiation with carbon dioxide. For each time point, three mice were used. The blood was collected into a heparinized syringe by cardiac puncture. Each blood sample was mixed with FIAU (10 μg) as internal standard, centrifuged at 12 800 rpm for 1 min, and the supernatant was taken for extraction by a Sep-Pak cartridge.[13] The Sep-Pak cartridge was preconditioned by washing with methanol (2 mL) followed by water (5 mL). The supernatant obtained above was loaded onto the conditioned cartridge, which was then washed by passing water (5 mL) through it. Methanol (2 mL) was used to elute the nucleosides/nucleoside derivatives. The methanol extract was dried under a stream of N_2 at 35 °C, the resultant residue was redissolved in methanol (50 μL), and an aliquot (20 μL) was subjected to HPLC analysis. HPLC analyses were performed using a Waters C_{18} cartridge (8 × 100 mm, 10 μm particle size) with a mobile phase of water:methanol (7:3, v/v) at a flow rate of 2 mL/min, with UV detection at 265 nm.

Biodistribution Studies. The biodistributions of analyte radioactivity resulting from [2-^{14}C]-labeled AZT, (5*S*,6*S*)- and (5*R*,6*R*)-BMAZT, and (5*R*,6*R*)-BEAZT were investigated after tail vein injection of each test compound (2.0 μCi in 100 μL of water) into Balb/c mice. At various intervals up to 8 h postadministration, the mice were sacrificed by CO_2 asphyxiation. A watch glass was used to collect urine excreted during sacrifice. The blood samples were collected by heart puncture. Several tissues including heart, liver, spleen, lung, kidney, blood, bone, brain, and bladder were collected and weighed (wet) in combustion cups. The weight of each tissue sample was limited to less than 200 mg (0.2 mL of blood). The tissues

were allowed to dry at room temperature prior to combustion in a biological oxidizer (Harvey Instrument Co). The $^{14}CO_2$ produced from the tissues by combustion was trapped in plastic scintillation vials containing carbon-14 trapping cocktail and the radioactivity was determined by liquid scintillation counting. Three mice were used for each time point.

Subcellular and Regional Distributions in Mouse Brain. The subcellular and regional distributions of analyte radioactivity after administration of [2-^{14}C]-labeled AZT, (5*R*,6*R*)-BMAZT, and (5*R*,6*R*)-BEAZT in brain were determined after jugular vein injection of the individual test compound (2 μCi in 100 μL water) into Balb/c mice. Ten minutes after injection, the mice were sacrificed, and the brains were removed.

For the subcellular distribution study, the brains were rinsed with a few drops of ice-cold sucrose solution (0.32 M) and the cerebella were removed. The remaining brain tissue was homogenized and subjected to centrifugation at 0−4 °C. The P_1 pellet (which contains nuclei and cell debris) was obtained by centrifugation of the crude homogenate at 1000*g* for 10 min (IEC B-20A centrifuge). The P_2 fraction [which contains myelin fragments, synaptosomes (pinched-off nerve endings) and mitochondria] and the P_3 fraction (microsomal fraction) were recovered as pellets after centrifugation at 10000*g* for 30 min and 100000*g* (Beckman 7−55) for 1 h, respectively. The final supernatant, S_3, was the soluble fraction. The protein content of each fraction was determined by the method of Lowry *et al.*[46] For the determination of radioactivity, the pellet fractions P_1, P_2, and P_3 were solubilized with basic solubilizer (Protosol), using a sample:Protosol ratio of 1:1, v/v, at 55 °C for about 30 min, followed by addition of glacial acetic acid (0.1 mL) to neutralize the samples and a few drops of freshly prepared 4% stannous chloride in 0.1 N HCl to quench chemiluminescence. The samples were then mixed with Aquasol-2 (15 mL) scintillation fluor. Fraction S_3 (2 mL) was directly mixed with Aquasol-2 (15 mL). The samples were counted in a liquid scintillation counter at a window setting for ^{14}C, with a preset time of 10 min. The counted samples were mixed with the ^{14}C standard (10 mL) [^{14}C]hexadecane (0.866×10^6 dpm/mL on July 1, 1988) and recounted. The counting efficiency thus obtained was used to correct the counting results from the samples.

For the regional distribution study, the brains were rinsed with ice-cold sucrose solution (0.32 M) and subsequently dissected into portions which included cerebellum, pons−medulla, hypothalamus, hippocampus, striatum, cortex, and the rest of brain. The specimens were dried at room temperature overnight and subjected to the combustion/liquid scintillation counting method for determination of radioactivity.

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