water bath) for about 2 min. The phosphorolysis reaction was initiated by adding the substrate solution (50 μL, 250 nmoles) to the prewarmed mixture. The contents of the closed tube were mixed well prior to a 10 min incubation at 37°C. The reaction was terminated by removing the tube from the water bath and adding ice-cold methanol (200 μl) with thorough Vortex mixing. The tube was placed in an ice bath for 10 min and then centrifuged at 12,300 rpm in an Eppendorf microcentrifuge for 3 min at 4°C. An aliquot of the clear supernatant was subjected to HPLC analysis or stored in a freezer for subsequent analysis. Each experiment was performed at least twice.

HPLC analyses were performed using a C_{18} Radial-PAK cartridge (8 mm I.D., 10 cm length, 10 μm particle size) with UV detection at 230 nm, using water:methanol concentration 7:3 as eluent at a flow rate of 2 mL/min.

Thymidine was used as a reference compound to confirm the phosphorolysis activity of the enzyme.

3.2.5. The stability of 5,6-dihydro analogs in phosphate buffer.

The stability of 5-halo-6-methoxy-5,6-dihydro analogs was investigated upon incubation of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with potassium phosphate buffer (pH 7.0, 0.06M) at a concentration of 1.0 mM at 37°C. After 1, 3, 5, 10 and 24 h, portions of 20 μL were removed from each reaction mixture and subjected to HPLC analysis.

HPLC analysis was performed on a reverse phase C18 cartridge using methanol:water (40:60) at 1 mL/min. The UV detector was set at 230 nm since the 5,6-dihydro analogs have low absorbance at 265 nm.

3.2.6. *In vitro* regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs.

The *in vitro* regeneration of parent drug from the corresponding 5-halo-6-methoxy-5,6-dihydro analogs was studied upon incubation of the test compounds with glutathione, mouse whole blood and the soluble enzyme fraction of mouse liver.

3.2.6.1. The regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs by glutathione.

The chemical regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs of AZT, d4T and FLT has been investigated by incubation of the individual test compound with glutathione (reduced) according to a literature method[231] with modifications as subsequently described.

Phosphate buffer (pH 7.0, 0.06 M) was prepared by dissolving potassium dihydrogen phosphate (0.8142 g) in distilled water (90 mL) and the pH was adjusted to 7.0 with 1.0 N KOH. The solution volume was then adjusted to 100 mL with distilled water. Glutathione solution was freshly prepared on the day of the experiment by dissolving glutathione in the phosphate buffer. The test substrate solution was also dissolved in phosphate buffer.

The test substrate solution was mixed with the glutathione solution using the molar ratios specified in the "Results and Discussion" Section. This mixture was then incubated at 37°C for various time periods up to 24 h. The resultant sample was analyzed by HPLC.

HPLC analyses were conducted using a C_{18} Radial-PAK Cartridge (8 mm I.D., 10 cm length, 10 μm particle size) with UV detection at both 230 nm and 265 nm using water:methanol 7:3 (v/v) as eluent at a flow rate of 2 mL/min.

The appearance of parent drug (AZT or FLT or d4T) at their known retention times, after the 5-halo-5-methoxy-5,6-dihydro analogs were incubated with glutathione,

indicated that regeneration of the 5,6-double bond had occurred. The % regeneration was determined using a standard curve prepared by plotting peak area vs concentration.

3.2.6.2. Regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs by mouse whole blood.

The regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs was studied by incubation of the individual test compound with heparinized blood from a Balb/c mouse.

Blood was drawn into a heparinized syringe from mice by heart puncture after the Balb/c mice were asphyxiated with carbon dioxide. The blood (100 μL) was transferred to each of the 1.5 mL microcentrifuge tubes and mixed with 100 μL of stock solution of test compound (1 mg/mL) followed by incubation at 37°C for 10 min. To terminate the reaction, the microcentrifuge tubes were removed from the 37°C water bath, and 200 μL of ice-cold methanol was added and the mixture was vortexed. The resultant mixture was kept in ice for 10 min before centrifugation at 12,800 rpm for 3 min in a cold room (4°C). The supernatant (200 μL) thus obtained was dried under a stream of N_2 at 35°C. The residue was then re-dissolved in 100 μL of methanol and an aliquot of 40 μL was subjected to HPLC analysis.

The HPLC analysis was performed using a reverse phase column C_{18} cartridge (8 mm I.D., 10 cm length, 10 μm particle size) with methanol:water (3:7) at 2 mL/min as eluent solvent. The UV detector was set to monitor absorbance at 230 nm and 265 nm.

3.2.6.3. Regeneration of parent drug from 5-halo-6-methoxy-5,6-dihydro analogs by the soluble enzyme fraction of mouse liver.

Preparation of soluble enzyme fraction of liver.

Three Balb/c mice were decapitated, and the livers were removed and washed with ice-cold 0.1 M Tris buffer (pH 8.2). The livers were homogenized in 2 volumes (w/v) of

the same buffer. The homogenate was centrifuged at 49,000 x *g* for 2 h at 4°C. The supernatant was then used to study the regeneration of parent drug from the corresponding 5,6-dihydro analogs.

Each individual test compound (100 µL, stock solution 1 mg/mL) was incubated with the soluble enzyme fraction of mouse liver (50 µL) at 37°C for half an hour. The reaction mixture was removed and mixed with an equal volume of methanol and subjected to centrifugation at 12,800 rpm for 3 min. The supernatant (200 µL) was dried under a stream of N_2 and redissolved in 100 µL of methanol. An aliquot of 25 µL was analyzed by HPLC method as described in Section 3.2.6.2.

3.2.7. Regeneration of AZT *in vivo* after i.v. administration of 5-halo-6-methoxy-5,6-dihydro analogs of AZT.

The *in vivo* regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs was studied in mice after i.v. injection of the individual test compound.

The test compound solution (3.6 µmol/0.1 mL) was prepared in 10% (v/v) DMSO/water. The solution was injected into male or female Balb/c mice via the tail vein. The mice were sacrificed at desired time periods by asphyxiation with carbon dioxide. For each time point, 3 mice were used. The blood was then drawn into a heparinized syringe by cardiac puncture.

Each of the blood samples was mixed with 10 µg of 1-(2'-fluoro-2'-deoxy-β-D-arabinofuranosyl)-5-iodouracil (FIAU) or 5-bromo-6-methoxy-5,6-dihydro-3'-fluoro-3'-deoxythymidine (FLTBrOMe) as internal standard and centrifuged at 12,800 rpm for 1 min, and the supernatant was taken for extraction by Sep-Pak™.[74] The Sep-Pak cartridge was first rinsed with 2 mL of methanol followed by 5 mL of water. The blood sample was loaded onto the conditioned cartridge, which was then washed by passing 5 mL of water through the device. 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 re-dissolved in 50 μL of methanol and an aliquot of 20 μL was injected into the HPLC for analysis. HPLC analysis was performed using a C_{18} cartridge 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 and 230 nm. Sensitivity for AZT and the 5,6-dihydro analogs was 0.1 μg/mL and 1 μg/mL, respectively. The recoveries of AZT, FIAU and FLTBrOMe by Sep-Pak extraction are 88%, 84% and 71%, respectively.

The amount of AZT in blood was quantitated using a standard curve which is a plot of peak ratio (AZT/FIAU or AZT/FLTBrOMe) vs. amount of AZT.

3.2.8. Biodistribution studies.

Biodistribution of [2-^{14}C]-AZT and its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs were investigated in Balb/c mice after i.v. injection.

3.2.8.1. Preparation of the samples.

The radiolabeled compounds, [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT (56 mCi/mmol, purity > 98%), were synthesized[303] from [2-^{14}C]-AZT which was purchased from Moravek Biochemical Inc. Two diastereomers of [2-^{14}C]-5-bromo-6-methoxy-5,6-dihydro analogs of AZT were separated by reverse phase HPLC using a Whatman Partisil ODS-3 (25 cm length) column, 80:20 water:methanol at 1.5 mL/min as eluent, with the UV detector set at 230 nm. The purified individual diastereomers were used for biodistribution studies. [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-, [2-^{14}C]-trans-(-)-(5S,6S)-5-bromo-6-methoxy-5,6-dihydro and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analogs of AZT were dissolved in sufficient water to make 20 μCi/ mL solutions.

3.2.8.2. Administration and biological sample collection.

A solution of test compound (100 μL; 2 μCi) was injected intravenously into each mouse via the tail vein. At different time intervals up to 8 h post administration, the mice were sacrificed by asphyxiation in a 800 mL beaker with carbon dioxide over which a watch glass was placed to collect excreted urine. Three mice were used for each time point. The blood was drawn by heart puncture. Several tissues including heart, liver, spleen, lung, kidney, blood, bone, brain and bladder were collected and weighed (wet) in the combustion cups. The weight of each tissue sample was limited to less than 200 mg with the exception of blood (0.2 mL) and urine, where varying volumes were taken. The tissues were allowed to dry at room temperature.

3.2.8.3. Sample analysis.

The biological tissues prepared by the above procedure were analyzed for ^{14}C by a combustion/liquid scintillation counting method, for which a Biological Oxidizer OX300 and a Beckman LS 9000 liquid scintillation counter were used. The catalyst zone temperature of the oxidizer was heated to 685°C and the combustion zone to 900°C during a 30 min period prior to use. The nitrogen and oxygen flows were adjusted to 350-400 mL/min and the time of combustion was set at 4 min for each sample. The $^{14}CO_2$ produced from the tissues by combustion was trapped in plastic scintillation vials containing Harvey carbon-14 cocktail and radioactivity was determined by liquid scintillation counting. The combustion efficiency was determined by combusting standard samples (^{14}C-hexadecane and/or ^{14}C-AZT) containing known amounts of ^{14}C and calculating the ratio of the CPM from a standard sample after combustion over the CPM of the same amount of standard sample without combustion. The counting efficiency was calculated as CPM/dpm of a known amount of ^{14}C-hexadecane. After achieving stable results with standard samples, tissue samples were then analyzed. All res t samples

were counted with a window setting for standard ^{14}C and a preset time of 10 min, after dark adaptation to eliminate chemiluminescence and phosphorescence.

3.2.9. Distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs in subcellular fractions of mouse brain.

A subcellular distribution study in mouse brain after jugular vein injection of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs was performed according to the literature procedure[311] with minor modifications.

3.2.9.1. Preparation of subcellular fractions of brain.

The brains obtained from 5 mice were rinsed with a few drops of ice-cold 0.32 M sucrose solution and the cerebella were removed. All of the other portions of the brains were weighed and placed in sufficient ice-cold 0.32 M sucrose solution to make a final homogenate concentration of 20% (w/v). The brain-sucrose solution was homogenized in a homogenizing tube by twelve reciprocal strokes with a Teflon plunger. The homogenization process was interrupted for about 1 min after the first six strokes to allow cooling by putting the tube in an ice-bath before continuing with the next six strokes. The resultant brain homogenate was then subjected to centrifugations at 0-4°C (shown in Figure 3.2.9.1). The P_1 pellet which contains nuclei and cell debris was obtained by centrifugation of the crude homogenate at 1000 x *g* for 10 min followed by two washes with the same sucrose solution using the same centrifugation conditions. The washings and the supernatant (S_1) were combined. The mixture was centrifuged at 10,000 x *g* for 30 min and the pellet was washed once. The resultant pellet P_2 contains myelin fragments, synaptosomes and mitochondria. The corresponding supernatant (S_2) and washing were combined and subjected to ultracentrifugation at 100,000 x *g* for 1 h to yield a microsomal pellet (P_3) and the supernatant S_3. All the fractions (P_1, P_2, P_3 and S_3) were frozen at -70°C for further analysis.

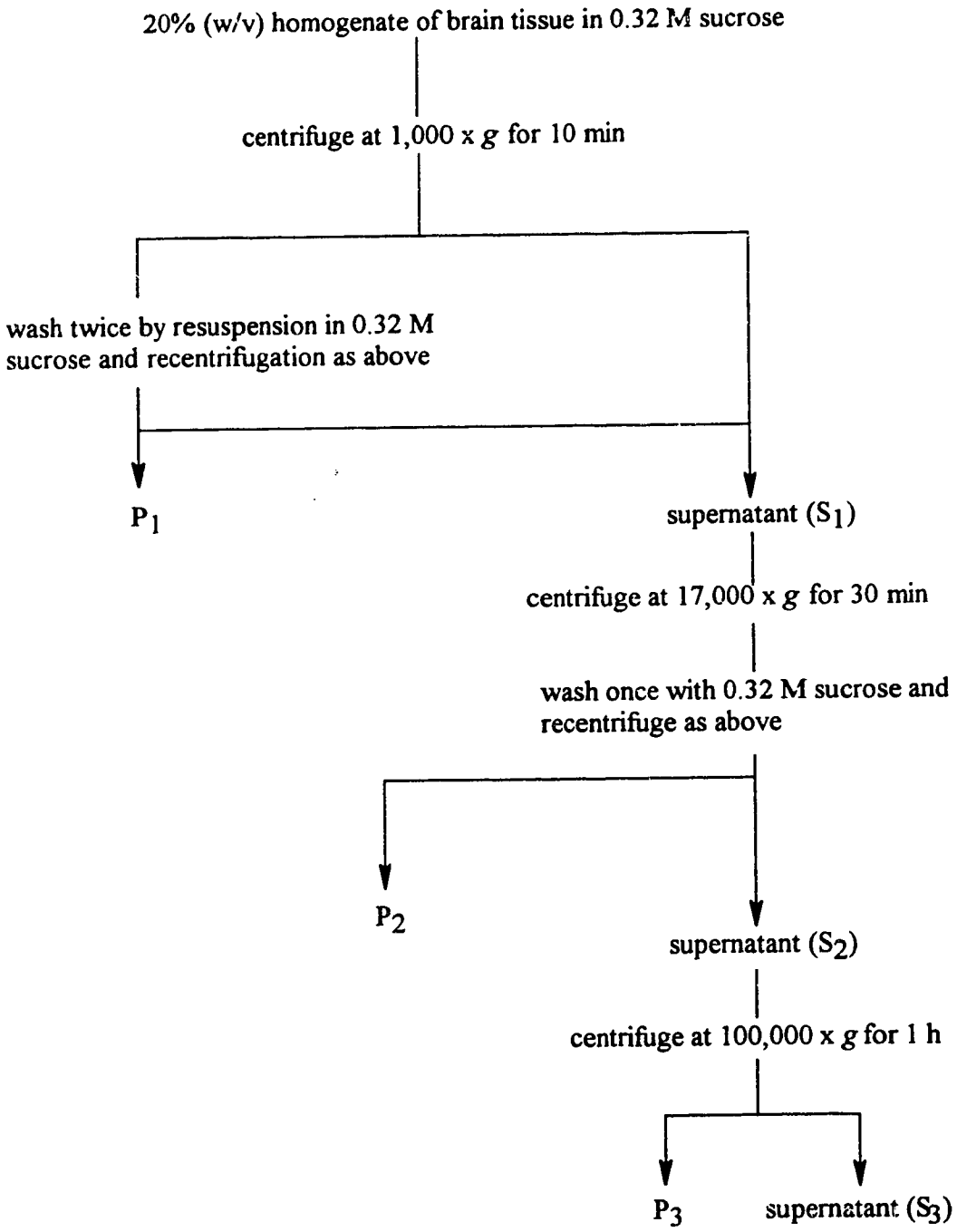


Figure 3.2.1. Flow diagram summarizing preparation of subcellular fractions of homogenized mouse brain.

3.2.9.2. Protein assay using the Lowry method.[312]

Preparation of reagents.

A. Bovine serum albumin (BSA). 1 mg/mL in distilled water was prepared as a stock solution. This solution can be prepared prior to use with storage at -70°C.

B. Membrane digestor. This solution was prepared by mixing 1 N NaOH and 1% Na deoxycholate (DOC) at a 1:1 ratio.

C Reagent A. Reagent A consists of the following constituents:
2% Na_2CO_3 : 1% $CuSO_4$: 2% NaKTartrate = 100 : 1 : 1 (v/v/v)

D. Folin Reagent (Folin-Phenol). A 1:1 mixture of commercial 2 N Folin and distilled water.

Solutions B, C and D must be prepared fresh just before use.

BSA is used to prepare the standard curve for analysis of protein contents in brain subcellular fractions. BSA (0-500 μL), or the sample to be analyzed, was added to the test tubes and the total volume was adjusted to 800 μL with distilled water. Membrane digestor (200 μL) was added to each of the tubes with thorough mixing. After 10 min, reagent A was added and mixed by vortex. After waiting for another 10 min, Folin reagent was added with vortex mixing. The absorbance at 660 nm was measured after at least 30 min and a blank sample (no BSA) was used as the reference. All BSA standards and samples were analyzed in duplicate.

The absorbance at 660 nm was plotted against the amount of BSA in the standard solution. The best fit line through the data points was calculated by linear regression. The amount of protein in the unknown sample was determined by interpolation from the standard curve. To get optimum absorbance, P_1, P_2 and P_3 had to be diluted by a factor of 5 with distilled water.

3.2.9.3. Radioactivity determination.

The pellet fractions P_1, P_2 and P_3 were solubilized with basic solubilizer (Protosol™) at a sample : Protosol ratio of 1:1, at 55°C for about 30 min, followed by addition of 0.1 mL of glacial acetic acid to neutralize the samples and a few drops of freshly prepared 4% stannous chloride in 0.1 N HCl. The samples were then mixed with 15 mL of Aquasol-2™ scintillation fluor. Fraction S_3 (2 mL) was directly mixed with 15 mL of Aquasol-2. 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 10 µL of ^{14}C standard, i.e. ^{14}C-hexadecane (0.866 x 10^6 dpm/mL on 1 July, 1988) and recounted. The counting efficiency thus obtained was used to correct the counting results from the samples.

3.2.9.4. Sample preparation and administration.

[2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro or [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analogs of AZT were each dissolved in sufficient water to make a 2 µCi/0.1 mL solution for use. The mouse was anesthetized with methoxyflurane by inhalation and monitored closely using the toe pinch reflex. Once the effective and painfree anaethesia was achieved, an incision of approximately 3/8" was made and the pectoral muscle and jugular vein were exposed. The test compound was injected via the jugular vein (2 µCi in 0.1 mL to each mouse). Ten minutes after the injection, the mice were sacrificed, and the brains were removed and chilled in an ice-cold petri dish. All subsequent steps were carried out as described in Section 3.2.9.1. The resultant P_1, P_2, P_3 and S_3 fractions were subjected to protein analysis and radioactivity determination.

The result for each individual fraction is expressed as total radioactivity, dpm/mg of protein as well as % radioactivity/%protein.

3.2.10. Regional distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of in mouse brain.

A study of the regional distribution of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analogs of AZT in brain was conducted in male Balb/c mice after jugular vein injection of the individual test compound.

The [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro or [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-ethoxy-5,6-dihydro analog of AZT was dissolved in sufficient distilled water to make a solution of 20 μCi/mL. The mouse was anesthetized with methoxyflurane using inhalation and monitored closely by the toe pinch reflex. Once effective and painfree anaethesia was achieved, an incision of approximately 3/8" was made and the pectoral muscle and jugular vein were exposed. [2-^{14}C]-AZT or its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs (0.1 mL) was injected via the jugular vein. Ten minutes after the injection, the mice were sacrificed, and the brains were removed and maintained in saline at ice bath temperature. The brain was subsequently dissected into portions which include cerebellum, pons and medulla, hypothalamus, hippocampus, striatum, cortex and the rest of brain. The samples were pooled from four (AZT) or two (5-bromo-6-alkoxy-5,6-dihydro analogs) mice after administration of the compound and weighed wet. The parts were dried at room temperature overnight and subjected to standard combustion/liquid scintillation counting method as described in Section 3.2.8.3. The results were expressed as CPM/mg for the specific samples.

4. Results and Discussion

A number of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T have been designed as new anti-HIV agents and as potential prodrugs to AZT, FLT and d4T for brain targeting.[313-315] The structures have been shown in Figures 1.5.13.-1.5.15. (pages 52-54). Four diastereomers are possible due to the presence of chiral centers at the C^5 and C^6 positions of the dihydrouracil ring. The configurations at C^5 and C^6 were assigned by comparison to literature NMR data and the acquisition of an X-ray crystal structure for (+)-(5R,6R)-5-chloro-6-methoxy-5,6-dihydro-1-(2',3'-didehydro-2',3'-dideoxy-β-D-glycero-2'-enopentofuranosyl)thymine.[316] The major diastereomers obtained in the 5-iodo-6-alkoxy and 5-bromo-6-alkoxy (or azido) reactions possessed the trans-(+)-(5R,6R) and trans-(-)-(5S,6S) configurations. In contrast, the cis-(+)-(5S,6R) and trans-(+)-(5R,6R) diastereomers were obtained in the 5-chloro-6-alkoxy (or azido) syntheses.

As prodrugs, the differences between these novel 5,6-dihydro analogs and the ester prodrugs previously reported in the literature are that these 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs have a) 5'-OH free, and b) modifications across the C^5, C^6 double bond.

4.1. *In vitro* cytotoxicities and anti-HIV activity of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T in HIV-1-infected CEM cells.

The *in vitro* anti-HIV activities and cytotoxicities of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T were evaluated by the U.S. National Cancer Institute Antiviral Evaluations Branch according to a literature method.[317] The ability of the test compound to protect HIV-1-infected T4 lymphocytes (CEM cells) from cell death

was determined. Small amounts of HIV are added to cells, and a complete cycle of virus replication is necessary to obtain the required cell killing. Agents that interact with virions, cells, or virus gene products to interfere with viral activities will protect cells from cytolysis. The EC_{50} value is the drug concentration which produces a 50% survival of HIV-infected cells relative to uninfected untreated controls and the IC_{50} value is the drug concentration which results in a 50% survival of uninfected untreated control CEM cells. The results are shown in Appendix I. The 5-iodo and 5-bromo-6-methoxy analogs exhibit EC_{50} values lower than their corresponding 5-chloro analogs with the EC_{50} values of 5-iodo-6-methoxy analogs generally similar to those of parent drugs. The results indicate that the relative *in vitro* anti-HIV activity of 5-halo-6-methoxy analogs is in the order of -I > -Br >Cl and the 5-iodo-6-methoxy analogs are approximately equipotent to their parent compounds. Among the 5-bromo-6-alkoxy analogs of AZT, the EC_{50} values generally increased when the size of the substituent at C^6 was increased or the alkoxy group becomes branched. However, the IC_{50} values remained about the same. In the case of the 5-chloro-6-alkoxy analogs of AZT, the compounds become inactive when the alkoxy groups at C^6 were bigger than ethoxy.[313-315] The results suggest that substituents at C^5 and C^6 are factors affecting the activity of the 5,6-dihydro analogs. The anti-HIV activities and cytotoxicities of these 5,6-disubstituted-5,6-dihydro analogs of AZT, FLT and d4T may reflect the activity of the individual compounds and/or may be related to the conversion of the 5,6-dihydro analogs to their parent compounds, since 5,6-dihydrouracils are known to convert to their parent compounds under various conditions.[231-233,318-323] The similarities between the activities of the parent drugs and their 5-iodo-6-methoxy-5,6-dihydro analogs may in fact be an indicator of fast regeneration of the parent drug. The decreased activities or inactivity observed for other 5-halo-6-alkoxy-5,6-dihydro analogs are likely due to slower regeneration or no regeneration of the parent drug. The 5-halo-6-methoxy-5,6-dihydro analogs of AZT were therefore selected for study of stability in the cell culture medium used for growth of CEM cells.

4.2. The stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT in MEMS/ 10%FBS.

Trans-(+)-(5R,6R)-5-chloro (bromo or iodo)-6-methoxy-5,6-dihydro analogs of AZT were incubated in the cell culture medium used for CEM cells, i.e., MEMS/10%FBS at a concentration of 1 mM at 37°C. After desired time periods, the samples were analyzed by HPLC. It was found that the trans-(+)-(5R,6R)-5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs of AZT gradually converted to AZT (Figure 4.2.1.), while the trans-(+)-(5R,6R)-5-chloro-6-methoxy-5,6-dihydro analog is stable, with no AZT formed after the 5-chloro-6-methoxy-5,6-dihydro analog was incubated in the cell culture medium for 48 h. These results suggest that 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs of AZT may have been partially converted to AZT under the conditions of determining the EC_{50} and IC_{50}. It therefore seems rational to conclude that the EC_{50} and IC_{50} values obtained do not reflect the activity of the 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs, since they are the results obtained from mixtures of the 5,6-dihydro analogs and AZT. However, the conversion from 5-chloro-6-methoxy-5,6-dihydro analog of AZT under the same conditions may be negligible, so the EC_{50} and IC_{50} values determined for the chloro analog are the results for this compound. The 5-chloro-6-methoxy-5,6-dihydro analog demonstrated increased EC_{50} and IC_{50} values relative to 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs, suggesting that this 5,6-dihydro analog is less potent than AZT. Thus the EC_{50} and IC_{50} observed for 5-iodo (bromo)-6-alkoxy-5,6-dihydro analogs of AZT, FLT and d4T may reflect the conversion from the dihydro analogs to their corresponding parent compounds.

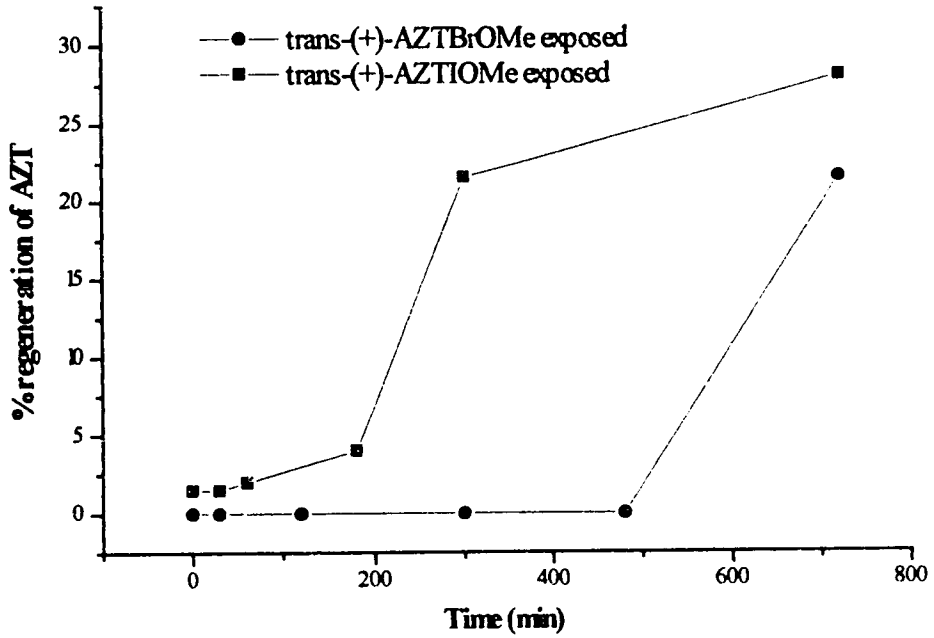


Figure 4.2.1. The regeneration of AZT from its 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs in MEMS/10%FBS at a concentration of 1 mM at 37°C.

4.3. Partition coefficients.

Strategies for drug delivery through the BBB involve the production of liposomes, drug lipidization and the development of lipid-soluble prodrugs, since the ability of a compound to traverse the cell membrane and the BBB is known to be at least partly dependent on the lipophilicity of the compound. However, liposomes have been generally ineffective because of their large sizes.[324] Our primary objective was to make prodrugs which are more lipophilic and therefore brain-targeted by virtue of their ability to diffuse readily across the BBB. The partition coefficient is a measure of lipophilicity and usually is considered to be an indicator of the ability of a compound to cross the cell membrane as well as the BBB into brain.[324,325]

Table 4.3.1. Partition coefficients (P) of thymidine, AZT and 5-halo-6-methoxy-5,6-dihydro analogs of AZT. Partition coefficients were determined in 1-octanol/water system at 37°C. $P = C_{1\text{-octanol}} / C_{water}$, where $C_{1\text{-octanol}}$ refers to the concentration of the test compound in the 1-octanol layer, and C_{water} refers to its concentration in the water layer after partitioning. The data shown are means ± SD, n = 5.

Compound	P value[a]
Thymidine	0.06[173]
Azidothymidine (AZT)	1.29 ± 0.12
trans-(+)-(5R,6R)-AZTClOMe	7.61± 2.81
cis-(+)-(5S,6R)-AZTClOMe	3.30 ± 1.10
trans-(+)-(5R,6R)-AZTBrOMe	13.21 ± 1.80
trans-(-)-(5S,6S)-AZTBrOMe	16.70 ± 5.23
trans-(+)-(5R,6R)-AZTIOMe	10.63 ± 1.41
trans-(-)-(5S,6S)-AZTIOMe	18.81 ± 1.13

The partition coefficients for AZT, FLT, d4T and their 5-halo-6-methoxy-5,6-dihydro analogs were determined in a 1-octanol/water system. The partition coefficient (P) is defined as $P = C_{1\text{-octanol}}/C_{water}$, at room temperature. The results, along with the reported partition coefficient for thymidine, are shown in Tables 4.3.1.- 4.3.3.

Compared with thymidine (P = 0.06)[173], AZT (P = 1.29), FLT (P = 0.5) and d4T (P = 0.12) are more lipophilic. Among them, AZT is the most lipophilic compound due to the $-N_3$ at the 3'- position of the sugar moiety. This is consistent with the fact that the azido substituent possesses very different physicochemical properties compared to the hydroxy group, especially steric size (molar refractivity value) and lipophilic (π-value) effects, which can be demonstrated from the results presented in Table 4.3.4.[326]

Table 4.3.2. Partition coefficients (P) of FLT and selected 5-halo-6-methoxy-5,6-dihydro analogs. Partition coefficients were determined in 1-octanol/water system at 37°C. $P = C_{1\text{-octanol}}/ C_{water}$, where $C_{1\text{-octanol}}$ refers to the concentration of the test compound in the 1-octanol layer, and C_{water} refers to its concentration in the water layer after partitioning. The data shown are means ± SD, n = 5.

Compound	P value
3'-Fluoro-3'-deoxythymidine (FLT)	0.50 ± 0.02
Trans-(+)-(5R,6R)-FLTClOCH$_3$	5.15 ± 0.28
Cis-(+)-(5S,6R)-FLTClOCH$_3$	1.50 ± 0.03
Trans-(+)-(5R,6R)-FLTBrOCH$_3$	4.71 ± 0.19
Trans-(-)-(5S,6S)-FLTBrOCH$_3$	3.44 ± 0.05
Trans-(+)-(5R,6R)-FLTIOCH$_3$	2.81 ± 0.09
Trans-(-)-(5S,6S)-FLTIOCH$_3$	4.00 ± 0.21

Table 4.3.3. Partition coefficients of d4T and selected 5-halo-6-methoxy-5,6-dihydro analogs. Partition coefficients were determined in 1-octanol/water system at 37°C. $P = C_{1\text{-octanol}} / C_{water}$, where $C_{1\text{-octanol}}$ refers to the concentration of the test compound in the 1-octanol layer, and C_{water} refers to its concentration in the water layer after partitioning. The data shown are means ± SD, n = 5.

Compound	P value
2',3'-Didehydro-2',3'-dideoxythymidine (d4T)	0.12 ± 0.02
Trans-(+)-(5R,6R)-d4TClOCH$_3$	2.60 ± 0.54
Cis-(+)-(5S,6R)-d4TClOCH$_3$	1.00 ± 0.14
Trans-(+)-(5R,6R)-d4TBrOCH$_3$	3.39 ± 0.29
Trans-(-)-(5S,6S)-d4TBrOCH$_3$	1.45 ± 0.12
Trans-(+)-(5R,6R)-d4TIOCH$_3$	2.60 ± 0.25
Trans-(-)-(5S,6S)-d4TIOCH3	1.25 ± 0.15

Table 4.3.4. Impact of -N_3 and -OH on molar refractivity and π values of a benzene ring.[327]

Substituent	Steric size effect (Molar refractivity value)	Lipophilic effect (π-value)
-N_3	10.2	+0.46
-OH	2.85	-0.67

The constant π was defined as $\pi_X = \log P_X - \log P_H$ by Fujita *et al*[327] and is used to correlate biological activity with chemical conposition. P_H is the partition coefficient of

a parent compound and P_X is that of a derivative of the parent compound with X as a substituent. A negative π value implies that the compound in question prefers the aqueous layer relative to the parent compound, and a positive π value indicates that the organic layer is preferred. By applying the same calculation to AZT and thymidine for the constant π (-N_3 vs. -OH), we found that π is equal to 1.30, which is close to the value of 1.13 attributed to their effects on a benzene ring.[327] The π value calculated for -F from FLT vs. thymidine is also determined as 0.90. To determine the π values of 5-halo and 6-methoxy, additional standard compounds (6-methoxy-5,6-dihydro and 5-halo-5,6-dihydro analogs) are needed. Molecular π values are therefore calculated relative to corresponding parent nucleoside analogs and are presented in Tables 4.3.5.-4.3.7. Trans-addition products generated similar π values (for AZT series, from 0.77 to 1.16; for FLT series, from 0.75 to 0.97 and for d4T series, from 1.08 to 1.45) while the cis-addition products have somewhat smaller effects.

Table 4.3.5. Impact of 5,6-disubstitutions on lipophilicity of AZT.

$\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of the 5,6-dihydro analog in question and P_H is the partition coefficient of AZT.

Substituent	π value
trans-(+)-(5R,6R)-5-chloro-6-methoxy	+0.77
cis-(+)-(5S,6R)-5-chloro-6-methoxy	+0.41
trans-(+)-(5R,6R)-5-bromo-6-methoxy	+1.01
trans-(-)-(5S,6S)-5-bromo-6-methoxy	+1.11
trans-(+)-(5R,6R)-5-iodo-6-methoxy	+0.92
trans-(-)-(5S,6S)-5-iodo-6-methoxy	+1.16

Table 4.3.6. Impact of 5,6-disubstitutions on lipophilicity of FLT.

$\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of the 5,6-dihydro analog in question and P_H is the partition coefficient of FLT.

Substituent	π value
trans-(+)-(5R,6R)-5-chloro-6-methoxy	+1.01
cis-(+)-(5S,6R)-5-chloro-6-methoxy	+0.48
trans-(+)-(5R,6R)-5-bromo-6-methoxy	+0.97
trans-(-)-(5S,6S)-5-bromo-6-methoxy	+0.84
trans-(+)-(5R,6R)-5-iodo-6-methoxy	+0.75
trans-(-)-(5S,6S)-5-iodo-6-methoxy	+0.90

Table 4.3.7. Impact of 5,6-disubstitutions on lipophilicity of d4T.

$\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of the 5,6-dihydro analog in question and P_H is the partition coefficient of d4T.

Substituent	π value
trans-(+)-(5R,6R)-5-chloro-6-methoxy	+1.34
cis-(+)-(5S,6R)-5-chloro-6-methoxy	+0.92
trans-(+)-(5R,6R)-5-bromo-6-methoxy	+1.45
trans-(-)-(5S,6S)-5-bromo-6-methoxy	+1.08
trans-(+)-(5R,6R)-5-iodo-6-methoxy	+1.34
trans-(-)-(5S,6S)-5-iodo-6-methoxy	+1.02

In 1954, Collander[328] demonstrated that the rate at which many organic compounds passed through the cellular material of Nittela cells was proportional to their log P ($P \leq 8.5$). This finding was confirmed by Milorrow and Williams in 1968.[329] However, Hansch and Fujita argued that the linear relationship could only be expected

when P is within a limited range.[327] A molecule partitions between many "aqueous" phases and "organic" phases in going through the wall-membrane section before it reaches the site of action. A compound with excessive lipid solubility will no longer circulate in the bloodstream, but rather bind to the first lipid membrane it encounters. Therefore, it has been known for a long time that as one increases the partition coefficient of a biologically active compound, biological activity often rises, but after a certain point it falls off and eventually reaches zero.[327] Thus there is often an optimum partition coefficient for a biologically active class of compounds,[330] as shown in Figure 4.3.1. P_a is defined as biological activity and P_0 is the optimal partition coefficient to achieve maximum biological effect.

It is known that the permeation of nucleosides through the BBB may be facilitated by increasing their lipophilicity, particularly for compounds with a molecular weight below 400.[331,355] The molecular weights of 5-halo-6-methoxy-5,6-dihydro analogs of AZT, FLT and d4T are in the range of 291 to 425 as shown in Table 4.3.8.

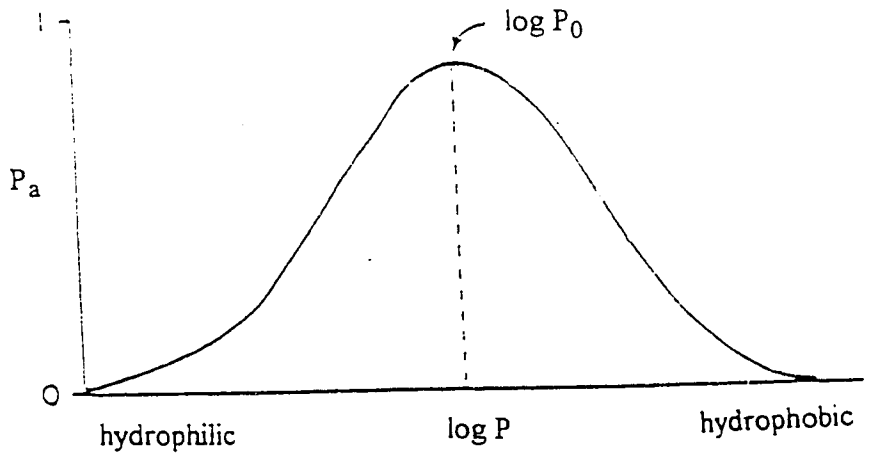


Figure 4.3.1. Relationship between biological activity (P_a) and lipophilicity (log P).

Table 4.3.8. Molecular weights of 5-halo-6-methoxy-5,6-dihydro analogs of AZT, FLT and d4T.

Substituent	Molecular Weight		
	AZT	FLT	d4T
5-Chloro-6-methoxy-5,6-dihydro	334	311	291
5-Bromo-6-methoxy-5,6-dihydro	378	355	335
5-Iodo-6-methoxy-5,6-dihydro	425	402	382

In this study, it has been found that all of the 5-halo-6-methoxy-5,6-dihydro analogs were more lipophilic than their corresponding 5,6-unsaturated parent compounds, i.e. AZT, FLT and d4T (Tables 4.3.1-4.3.3). The P values for the 5-halo-6-methoxy-5,6-dihydro analogs of AZT were 2.5 to 15 times greater than that of AZT. The P values of 5-halo-6-methoxy-5,6-dihydro analogs of FLT were 3 to 10-fold higher than that of FLT, and the 5-halo-6-methoxy-5,6-dihydro analogs of d4T were 8 to 28 times more lipophilic than d4T. The two diastereomers of each analog also possess different lipophilicities. Among the 5,6-dihydro analogs, cis-(+)-(5S,6R)-5-chloro-6-methoxy-5,6-dihydro analog has the lowest partition coefficient relative to all other corresponding trans-5,6-dihydro analogs. This is likely due to the larger dipole-dipole effect across the C^5 and C^6 chemical bond as the result of the cis- addition compared to other trans- analogs. These results indicate that the configurations of the C^5 and C^6 chiral centers in the two diastereomers are determinants of lipophilicity. These results are similar to a previous report on 5-halo-5-ethyl-6-methoxy-5,6-dihydro analogs of 5-ethyl-2'-deoxyuridine (EDU), which were shown to be more lipophilic (5- to 35-fold) than their parent drug, EDU.[232]

The relative lipophilicities of these compounds can also be observed from their retention times on reverse-phase HPLC chromatography. The more polar the compound, the smaller the retention time. The retention times of AZT and its 5-halo-6-methoxy-5,6-dihydro analogs are summarized in Table 4.3 9. Balzarini *et al*[332] reported that there was a correlation (correlation coefficient > 0.970) of P with t_R (retention time on HPLC) values for 36 anti-HIV nucleoside analogs that they investigated. Their results suggest that there are close linear correlations between the log P and t_R values of pyrimidine 2',3'-dideoxynucleosides, purine 2',3'-dideoxynucleosides, and 5-halogeno-substituted 3'-fluoro-2',3'-dideoxynucleosides. Correlation of log P with t_R for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs also shows a linear relationship (Figure 4.3.2.) that is described by the following equation:

$$\log P = 0.061\ t_R - 0.2215 \quad \cdots\cdots\cdots\cdots \text{Equation 4.3.1.}$$

The correlation coefficient (R^2) for this relationship is 0.90. The linear relationship derived from 5-halo-6-methoxy-5,6-dihydro analogs of AZT may provide a useful tool to predict the partition coefficients of other 5-halo-6-alkoxy-5,6-dihydro analogs from their retention times under the same HPLC conditions. Although the P values for other 5-halo-6-alkoxy (other than methoxy)-5,6-dihydro analogs, were not determined, one can assume that elongation of the C^6 alkoxy chain increases the P-values.

According to Hansch and Fujita[327], there should be an optimum log P (log P_0) for achieving the best biological activity. It has been shown previously[355] that log P's between 0.9 and 2.5 are optimal for brain extractability of certain radiopharmaceuticals. However, without further studies employing additional compounds, this optimum log P_0 cannot be defined for the nucleosides used in the current study.

Table 4.3.9. Retention times of AZT and its 5-halo-6-methoxy-5,6-dihydro analogs on reverse phase HPLC. Column: Partisil 5, ODS-3, 25 cm; Mobile phase: methanol: water (60:40, v/v); flow rate: 1 mL/min; UV detection at 230 nm with the exception of 265 nm for AZT.

Compound	Retention time (min)
Azidothymidine (AZT)	7.5
cis-(+)-(5S,6R)-AZTClOMe	10.0
trans-(+)-(5R,6R)-AZTClOMe	21.0
trans-(+)-(5R,6R)-AZTBrOMe	20.5
trans-(-)-(5S,6S)-AZTBrOMe	24.0
trans-(+)-(5R,6R)-AZTIOMe	18.7
trans-(-)-(5S,6S)-AZTIOMe	24.0

It has been reported that AZT crosses the cell membrane of human lymphocytes by a nonfacilitated diffusion process (passive diffusion) and its uptake is insensitive to inhibitors of nucleoside transport.[173] Its unusual ability to cross the cell membrane is thought to be related to its partition coefficient, which is higher than that of other nucleosides.[173] Based on the previous findings that increased lipophilicity usually favors passive diffusion across cell membranes, it is postulated that the enhanced lipophilicity observed for the 5,6-dihydro analogs will increase the cellular uptake significantly. Other studies have demonstrated that prodrugs with increased lipophilicities possessed enhanced cellular uptake.[206,291] DP-AZT with a partition coefficient of 4.8 resulted in a 50% increase in cellular uptake compared with AZT.[291] Most 5'-ester prodrugs with higher partition coefficients (7.2-17.9) reported by Aggarwal *et al*[206] demonstrated increased cellular uptake (up to four-fold higher than AZT) and 5'-amino acid analogs of AZT with

decreased partition coefficients showed reduced ability to cross cell membranes. These results suggest that these 5'-amino acid analogs of AZT may not be transported by the active transport system used for amino acids and that the partition coefficient plays an important role in the diffusion of these AZT analogs into cells.

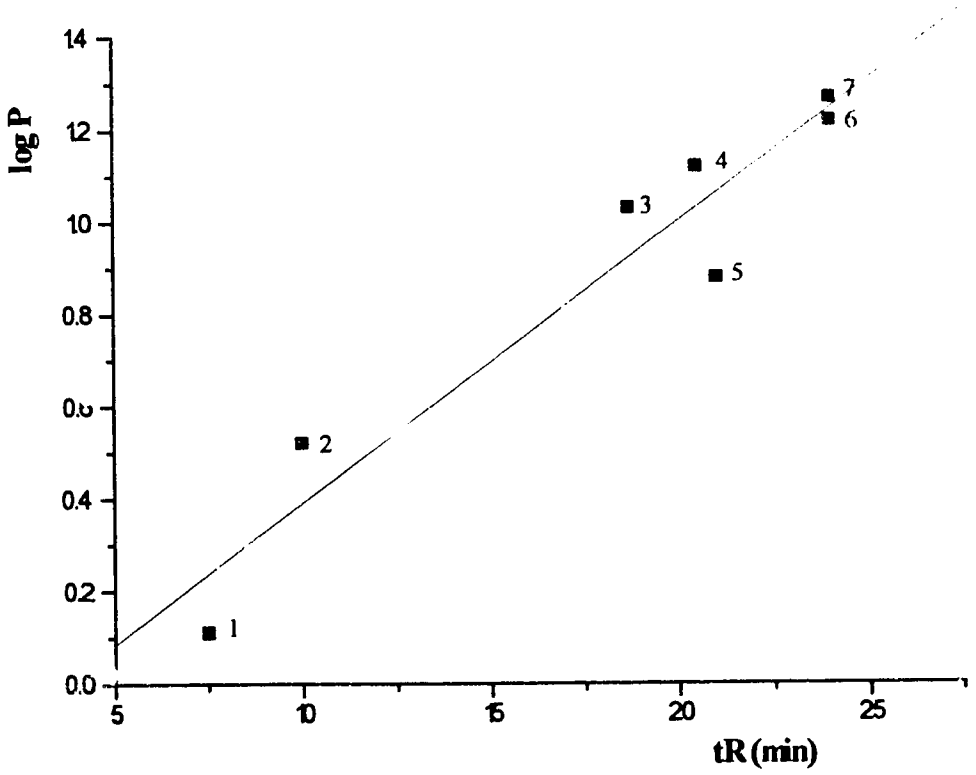


Figure 4.3.2. The correlation between lipophilicity (log P) and retention time (t_R) for reverse phase HPLC (Column: Partisil 5, ODS-3, 25 cm; Mobile phase: methanol: water (60:40, v/v); flow rate: 1 mL/min; UV detection at 230 nm with the exception of 265 nm for AZT.) 1 = AZT, 2 = cis-(+)-(5S,6R)-AZTClOMe, 3 = trans-(+)-(5R,6R)-AZTIOMe, 4 = trans-(+)-(5R,6R)-AZTBrOMe, 5 = trans-(+)-(5R,6R)-AZTClOMe, 6 = trans-(-)-(5S,6S)-AZTBrOMe, 7 = trans-(-)-(5S,6S)-AZTIOMe.

4.4. Interaction of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with the NBMPR-sensitive nucleoside transport system in mouse erythrocytes.

Many nucleosides traverse cell membranes by facilitated mechanisms. However, AZT and ddNs such as d4T, ddI and ddA, which lack the 3'-OH moiety and are more lipophilic, may cross cell membranes mainly or partially by passive diffusion.[173,177-180] FLT has been shown to cross the cell membrane by two mechanisms, NBMPR-sensitive and NBMPR-insensitive processes.[333]

The importance of influx and efflux of ddNs, including AZT through membrane as initial determinants of their intracellular anabolism has been recognized. Moreover, it has been reported that dipyridamole (an inhibitor of nucleoside transport) potentiated the activity of AZT in monocyte/macrophage due at least in part to suppression of thymidine transport and phosphorylation, which resulted in increased phosphorylation of AZT.[334,335] On the other hand, coadministration of probenecid with AZT has provided elevated CSF/plasma ratios of AZT in both human and animals because of the inhibition of efflux of AZT from CSF.[210-212]

The objective of this study was to investigate the interaction of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with the NBMPR-sensitive NT system, which is used by thymidine to cross human erythrocyte membranes. Due to the unavailability of radiolabeled 5,6-dihydro analogs which can be used for direct cellular uptake studies, thymidine influx competition experiments in which only radiolabeled thymidine is needed, were performed. Mouse erythrocytes were used as a simple model.

4.4.1. Thymidine influx time course in fresh murine erythrocytes.

The objective of this experiment was to determine the kinetic characteristics of thymidine influx across the cell membrane via the NBMPR-sensitive transport system in $B_6D_2F_1$ mouse erythrocytes at room temperature. It is known that metabolism of

thymidine does not occur in the mouse erythrocyte, so the erythrocyte provides an uncomplicated model for uptake studies.[306] It is also known that this transporter has a broad permeant specificity.[305,306,336,337]

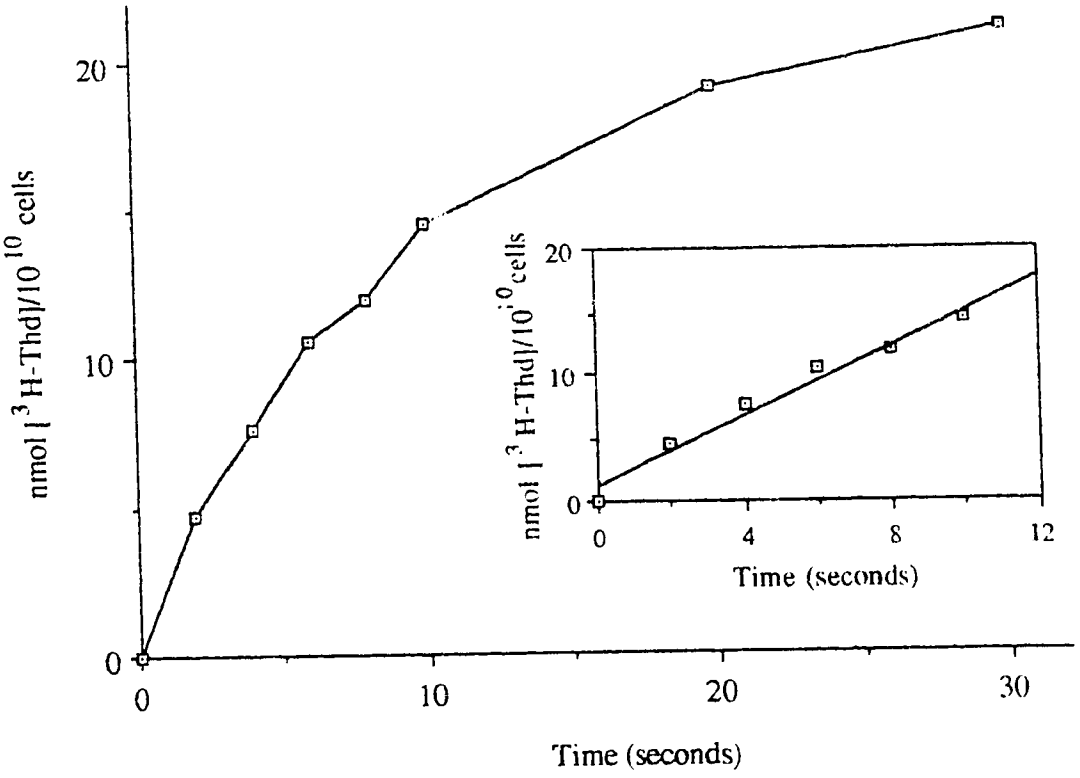


Figure 4.4.1. Time course of thymidine uptake in fresh murine erythrocytes. The intervals of thymidine uptake were initiated by the addition of 285 μM [^{3}H]-thymidine (0.2 mL) to equal volume of cell suspensions (3 x 10^8 cells). Fluxes intervals were terminated by the addition of NBMPR solution at a final concentration of 10 μM. The data shown are means of three replicate determinations.

The time course for thymidine influx was studied by incubating ^{3}H-thymidine with mouse erythrocytes, and stopping the transport process at specified time periods by the addition of an NBMPR solution. The transport rate was determined by measuring the intracellular concentration of ^{3}H-thymidine using the liquid scintillation method. The initial thymidine influx appears linear (see Figure 4.4.1.), but it became non-linear after 10 s. The proper use of NBMPR was essential in order to determine initial velocity of thymidine influx. In consideration of the technique of handling, a 3 s time interval was selected to determine the initial uptake rate for subsequent influx competition studies.

4.4.2. Determination of K_m and V_{max} for thymidine influx.

The kinetics of thymidine influx across mouse erythrocytes can be described by Michaelis-Menten kinetics,[338-340] in which the system can become saturable with thymidine. K_m is defined as the Michaelis constant, or half-saturation constant, and V_{max} is the maximum velocity. K_m and V_{max} values for the zero-trans influx of thymidine have always been measured in the absence of a competing permeant when each influx competition study was performed. Using the method of Hanes (Figure 4.4.2.),[307] the data plotted in Figure 4.4.3. provided a K_m = 0.076 mM, V_{max} = 5.81 nmoles/10^{10}cells/s. These values are comparable to the reported values (K_m = 0.075 mM, V_{max} = 6.25 nmoles/10^{10}cells/s).[341]

4.4.3. Determination of inhibition constants for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs.

Although the NBMPR-sensitive NT system accepts a variety of compounds,[186] limitations on structural modifications tolerated by the transporter, especially on the sugar moiety, have been reported.[202,305,306,341] Decreased affinity has been demonstrated with dideoxythymidine suggesting that 3'-OH plays an important role in binding of pyrimidine nucleosides to the transporter. It has also been demonstrated that the replacement of 3'-

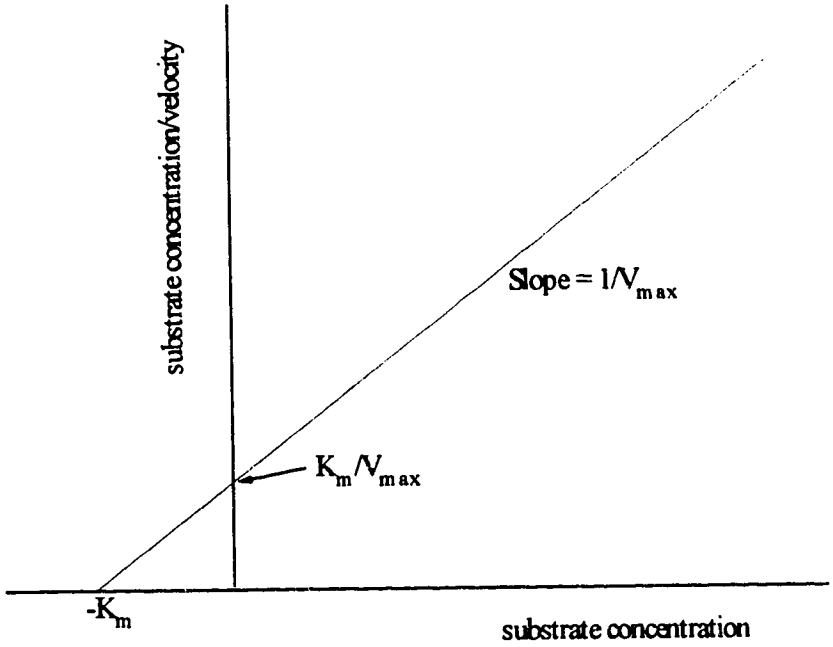


Figure 4.4.2. Hanes plot (method for determining K_m and V_{max}).

OH with a halogen is considerably less acceptable than replacement with a hydrogen. As reported previously, AZT and some other ddNs, such as d4T and ddA, cross cell membranes predominantly by simple diffusion due to the lack of a 3'-OH group and the higher lipophilicity.[173,177,306] However, the interaction between 5,6-dihydro uracil analogs and the NBMPR-sensitive nucleoside transport system has not been reported. Since thymidine transport inhibitors are known to enhance AZT anti-HIV activity,[342] we were prompted to investigate the interaction of 5-halo-6-methoxy-5,6-dihydro analogs of AZT with the thymidine transport system by performing zero-trans influx competition studies. The inhibition constant (K_i) can be obtained using the Dixon method[308] (Figure 4.4.4). An example is shown in Figure 4.4.5. For all the test compounds, the lines intersected above the [i]-axis, indicating a competitive inhibition of thymidine influx.[343] The value of K_i represents the relative affinity of the test compound for the external sites

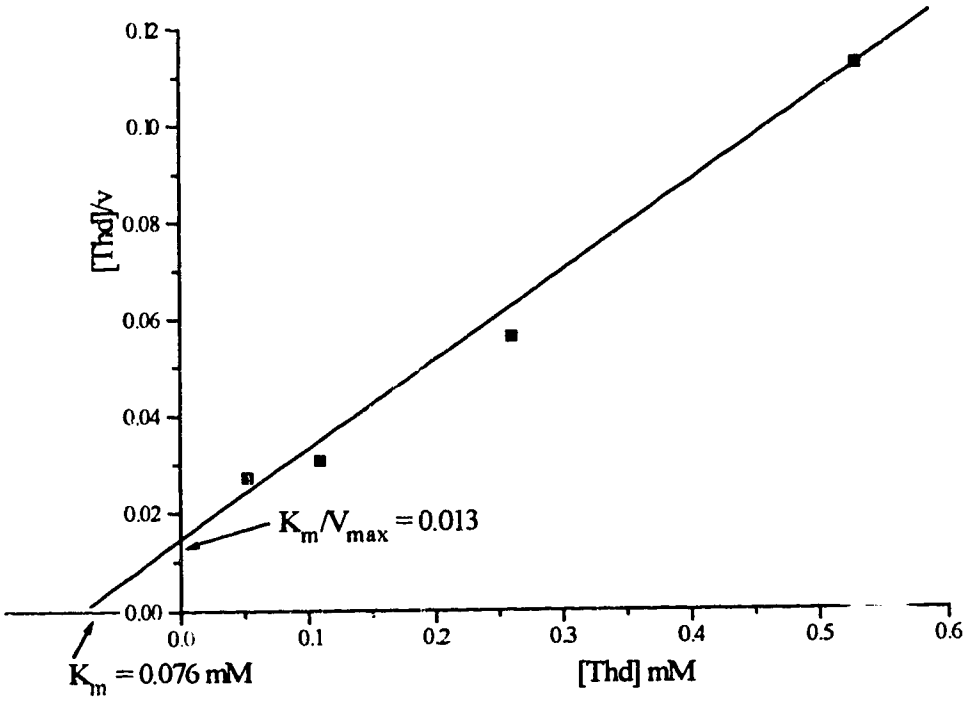


Figure 4.4.3. Determination of K_m and V_{max} for thymidine influx in fresh mouse erythrocytes. The influxes were measured in cell suspensions (3 x 10^8 cells) after 3 s of permeant exposure. The data shown are means of three replicate determinations.

on the NBMPR-sensitive nucleoside transport system. The larger the K_i value, the lower the affinity of the test compound for the transporter. The K_i values for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs determined are presented in Table 4.4.1.

The K_i for AZT (see Table 4.4.1) was determined to be 1.33 mM, which is comparable to the previously reported value.[173] With increased lipophilicity that favors passive diffusion and the absence of a 3'-OH moiety which appeared to be an essential component for the transporter system,[305,306,341] less interaction would be expected from the 5,6-dihydro analogs of AZT. However, it was found that all of the trans- products behaved very similarly, with K_i values in the 0.2-0.4 mM range, indicating that these trans-

5,6-dihydro analogs have an increased affinity for the transporter relative to AZT. On the other hand, cis-(+)-(5S,6R)-AZTClOMe showed a significantly increased K_i value of greater than 1.5 mM, indicating a decreased affinity for the NT system due to unfavorable steric effects. This is likely because the trans- addition of halogen and methoxy to the 5,6-double bond renders the molecule more complimentary to the binding site since binding of the compound to the transport system is likely stereospecific.[204] With the increased affinity for the transporter observed with the trans- 5,6-dihydro analogs, there are two possible interpretations. One possibility is that these 5,6-dihydro analogs are actually transported by the transporter. The other possibility is that they are not transported, but that the decreased K_i value is just the result of non-specific lipophilic binding of the test compounds to the transport system. However, to resolve this problem, more experiments are needed. It is essential to do an efflux experiment to determine whether the NBMPR-sensitive NT system is involved in the membrane transport of these 5,6-dihydro analogs. In the efflux experiment, trans-acceleration of efflux provides evidence that the test compound is translocated across the membrane by the nucleoside transporter. Thus, the method we used does not provide direct information regarding the exact mechanism by which the test compound is transported. The important information obtained from this experiment is that all trans- addition products, regardless of the specific halogen atom, have a higher affinity relative to AZT for the NBMPR-sensitive thymidine transport system (increased interaction between these 5,6-dihydro analogs and the transport system), and the cis-(+)-(5S,6R)-AZTClOMe exhibited a decreased affinity for the same transport system. These results suggest that the configurations of the C^5 and C^6 chiral centers play an important role in determination of the affinity of 5,6-dihydro analogs for the mouse erythrocyte NBMPR-sensitive nucleoside transport system. Irrespective of whether the 5,6-dihydro analogs are transported by the NBMPR-sensitive transport system or not, the trans- 5,6-dihydro analogs do inhibit the transport of thymidine more effectively than AZT.

Table 4.4.1. Thymidine influx inhibition constants (K_i) for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs in murine erythrocytes. Influxes of [^{3}H]-thymidine were measured in cell suspensions (3 x 10^8 cells) after 3 s of permeant exposure, in the absence or presence of graded concentrations of individual test compound. Influxes were initiated by the addition of the permeant solutions to the cell suspensions and terminated by the addition of NBMPR solution at a final concentration of 10 μM. The data shown are means from three experiments ± SD.

Compound	Inhibition constant (K_i)
AZT	1.33 ± 0.04 mM
cis-(+)-(5S,6R)-AZTClOMe	>> 1.5 mM
trans-(+)-(5R,6R)-AZTClOMe	0.36 ± 0.01 mM
trans-(+)-(5R,6R)-AZTBrOMe	0.45 ± 0.07 mM
trans-(-)-(5S,6S)-AZTBrOMe	0.50 ± 0.04 mM
trans-(+)-(5R,6R)-AZTIOMe	0.35 ± 0.03 mM
trans-(-)-(5S,6S)-AZTIOMe	0.20 ± 0.03 mM

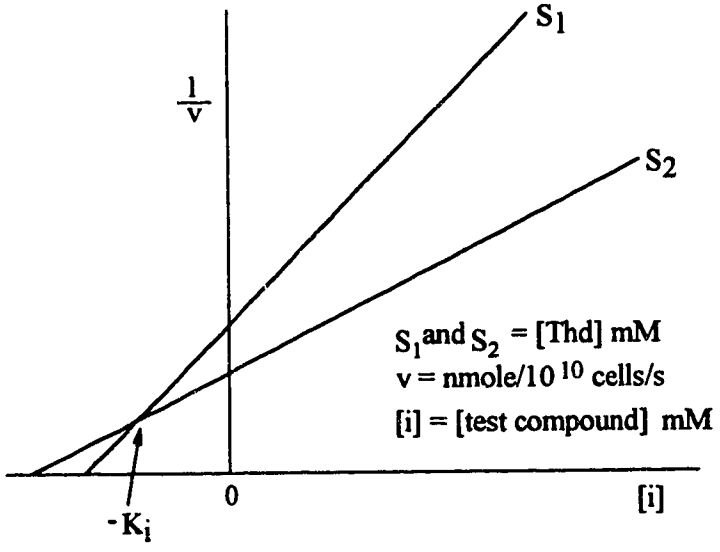


Figure 4.4.4. Dixon plot (method for determining K_i).

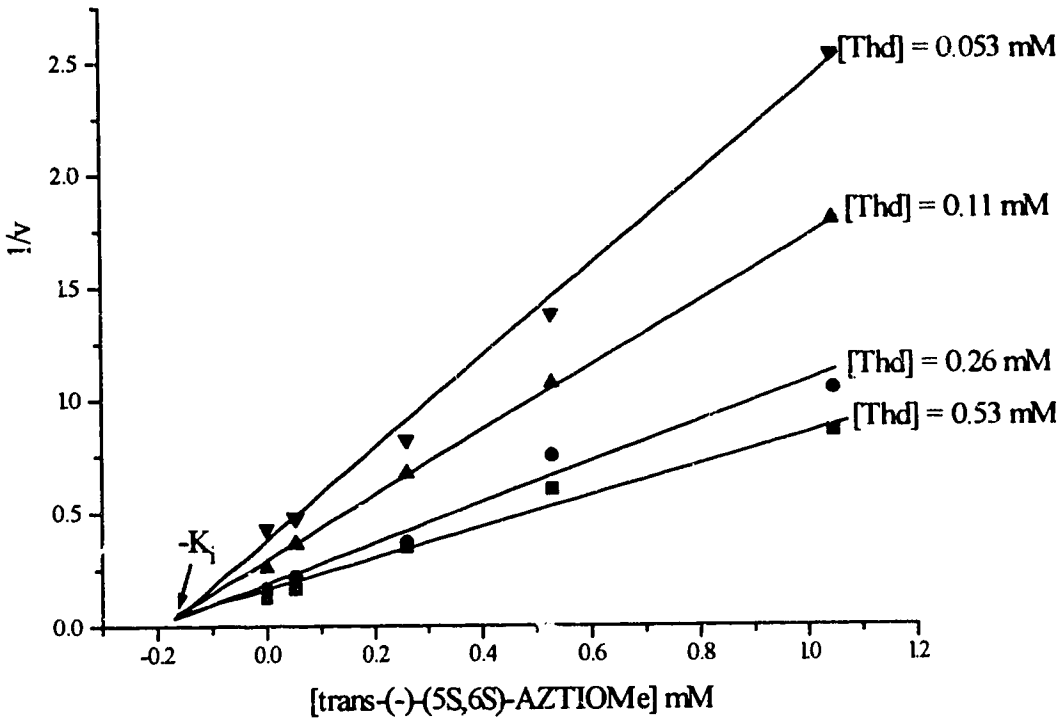


Figure 4.4.5. Determination of the K_i for trans-(-)-(5S,6S)-AZTIOMe. Influxes of [^{3}H]-thymidine were measured in fresh mouse erythrocytes (3 x 10^8 cells) after 3 s of permeant exposure, in the absence or presence of graded concentrations of trans-(-)-(5S,6S)-AZTIOMe. Influxes were initiated and terminated in response to metronome signals. NBMPR (20 μM) was used as the inhibitor to stop the influxes.

According to previous findings that a thymidine transport inhibitor increased the activity of AZT,[342] the 5,6-dihydro analogs we have studied are important. These nucleosides may exhibit double functions. They have better permeation ability because of their enhanced lipophilicity and on the other hand they reduce the permeation of thymidine into the cell because of their increased affinity for the NBMPR-sensitive transport system.

Although a number of AZT prodrugs (5'-esters) have been proposed, the interaction of those prodrugs with nucleoside transporters have not been reported.

4.5. *In vitro* stability of the 5-halo-6-methoxy 5,6-dihydro analogs in the presence of thymidine phosphorylase.

As an anti-cancer or anti-viral agent, a nucleoside must undergo anabolism to its 5'-triphosphate, and it is the latter which is responsible for its biological functions. However, for many nucleoside analogs, phosphorolysis (catabolic cleavage of the glycosidic bond with the formation of nucleobase and 1-phosphate ribose or 1-phosphate-2'-deoxy-ribose, shown as follows) is a competing process which causes degradation of the nucleoside.[344-347]

$$\text{Pyr-(d)R} + \text{Pi} \rightleftharpoons \text{Pyr} + \text{(d)-R-1-P}$$

Two pyrimidine nucleoside phosphorylases, uridine phosphorylase and thymidine phosphorylase, exist in the cytosol of mammalian cells and catalyze this reversible phosphorolysis of a numbe. of naturally occurring and synthetic pyrimidine nucleosides. Thymidine phosphorylase is specific for the 2'-deoxyribosyl moiety of nucleosides, whereas uridine phosphorylase has a broader substrate specificity. Many pyrimidine nucleosides (e.g. uridine and 5-fluorouridine, FUDR) which do not bind to thymidine phosphorylase are good substrates for uridine phosphorylase.[344,348-351] 5-Substituted-2'-deoxyuridines are also better substrates for uridine phosphorylase than for thymidine phosphorylase. As an example, it has been found that the anabolism of FUDR (phosphorylation to corresponding 5'-phosphate-FUDR) is largely preempted by the rapid catabolism to FU followed by catabolic breakdown of the latter; this process is an important factor responsible for the decreased potency observed for FUDR *in vivo*.[344]

As prodrugs, it is important that the 5,6-dihydro analogs do not undergo catabolic cleavage, so that active drug concentrations can be attained (Figure 4.5.1.). Phosphorolysis of the prodrugs will result in a decrease in bioavailability, and stability towards phosphorolysis will allow the buildup the active drug *in vivo*.

Although a previous report indicated that AZT was not a substrate for either thymidine phosphorylase or uridine phosphorylase and that it only binds to uridine phosphorylase, d4T has been reported to be a good substrate of thymidine phosphorylase. On the basis of these data, it is postulated that the 5,6-dihydro analogs of d4T may provide greatly improved stability *in vivo*. Since AZT is already a poor substrate for phosphorolysis, the advantage of the 5,6-dihydro analogs may be less important. The susceptibility of the 5-halo-6-alkoxy-5,6-dihydro analogs of AZT, d4T and FLT for the phosphorolysis has not been reported. It was therefore considered important to investigate the stability of these prodrugs toward phosphorolysis.

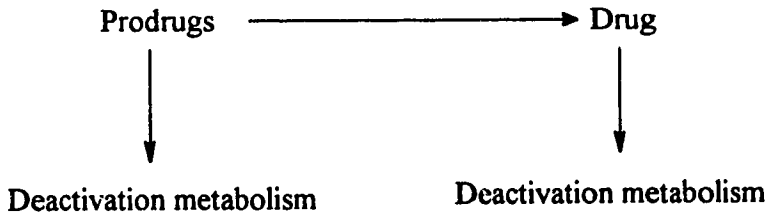


Figure 4.5.1. The possible metabolic pathways of the prodrugs.

An *in vitro* enzymatic study using *E. coli* thymidine phosphorylase provides a simple model for studying this process. *E. coli* thymidine phosphorylase is specific for pyrimidine deoxyribonucleosides.[310] Purine deoxyribonucleosides and ribonucleosides as well as deoxycytidine are not cleaved by this enzyme. The enzyme is stable for several months when kept properly. The stability of the enzyme is dependent on temperature and protein concentration. Thymidine phosphorylase from mammalian tissues has been reported to demonstrate similar substrate specificity to the bacterial enzyme.[348] This *in*

vitro phosphorolysis study was carried out by incubating the individual test compound with *E. coli* thymidine phosphorylase which is commercially available.

Thymidine was incubated with *E. coli* thymidine phosphorylase at 37°C and after specified time intervals (5, 10, 20, 30, 60 and 360 min), analysis was performed by HPLC to determine the degree of phosphorolysis. As illustrated in Figures 4.5.2., the process of phosphorolysis can be monitored by HPLC. The phosphorolysis of thymidine yielded thymine, which was confirmed by comparing the retention time of the products with that of a reference standard of thymine. The thymidine phosphorolysis time course is shown in Figure 4.5.3. It is clear that phosphorolysis reached its maximum after 10 min, and therefore 10 min was chosen for all phosphorolysis studies of AZT, d4T and their 5-halo-6-methoxy-5,6-dihydro analogs as well as other nucleoside analogs. The individual test compound (250 nmol) was incubated with thymidine phosphorylase (0.2 u) for 10 min at 37°C. The sample was analyzed by HPLC. The sensitivity of UV detection for the 5,6-dihydro analogs was approximately 1 μg/mL. As presented in Table 4.5.1., it was found that AZT does not undergo catabolic cleavage by *E. coli* thymidine phosphorylase. The 5-halo-6-methoxy-5,6-dihydro analogs of AZT have retained this stability. Other nucleoside analogs tested, including 2',3'-dideoxy-2',3'-didehydrothymidine (d4T), 2'-deoxyuridine (DU), and 5-ethyl-2'-deoxyuridine (EDU), underwent 10%, 57% and 72% phosphorolysis, respectively, whereas their 5-halo-6-methoxy (or azido)-5,6-dihydro analogs were found not to be susceptible to phosphorolysis by *E. coli* thymidine phosphorylase. The results suggest that the 5,6-disubstituted-5,6-dihydro pyrimidine analogs are not substrates for *E. coli* thymidine phosphorylase irrespective of whether their parent pyrimidines are, or are not, substrates for the enzyme. The stability against *E. coli* thymidine phosphorylase of the test compounds *in vitro* may suggest that they are stable in the blood circulation. However, the stability of these compounds upon incubation with uridine phosphorylase, which has a broader substrate specificity than thymidine phosphorylase, was not studied.

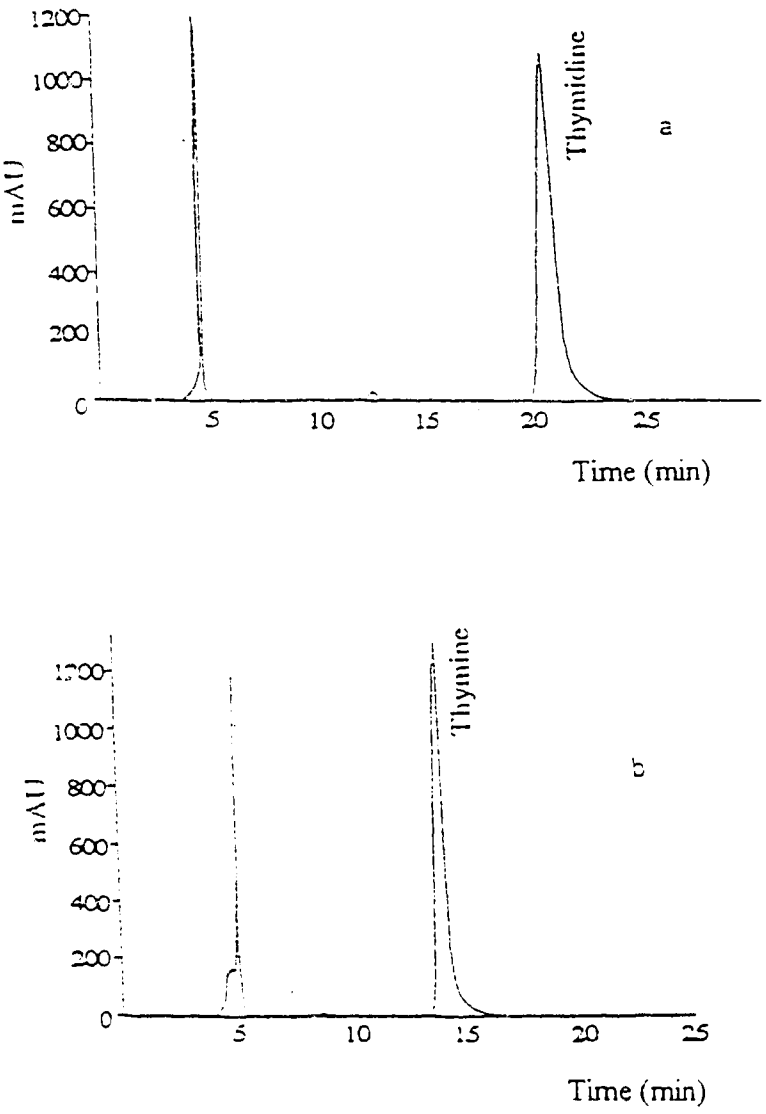
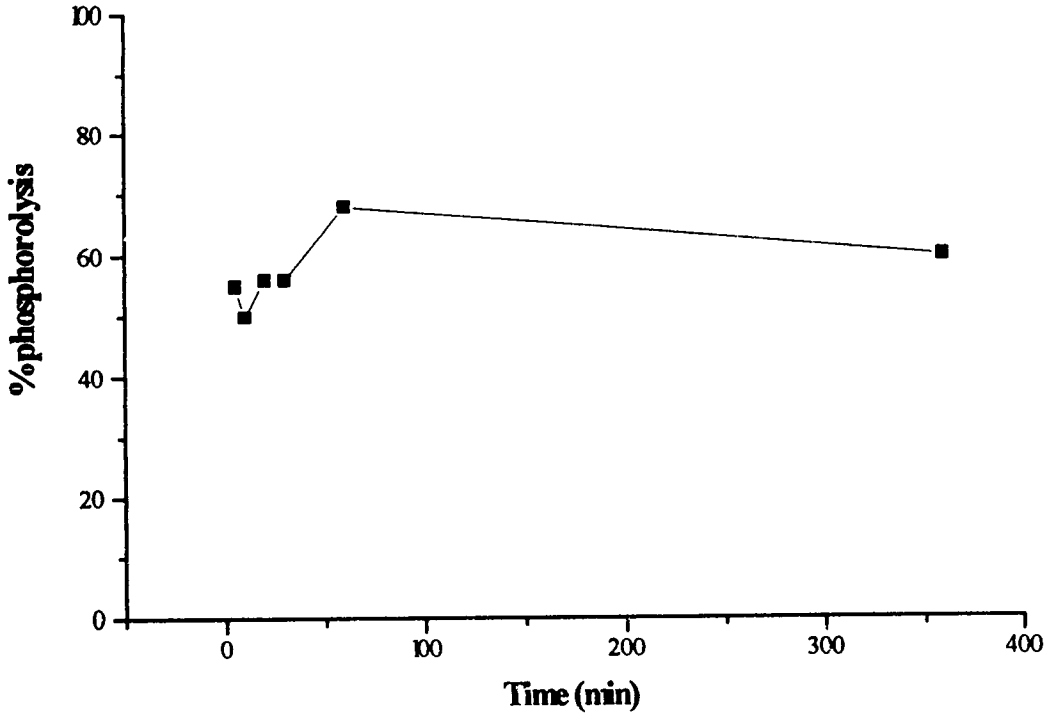


Figure 4.5.2. HPLC chromatograms for **a.** thymidine (retention time = 20 min) and **b.** thymine (retention time =14 min) using a Whatman partisil ODS column (25 cm) with water:methanol 9.5:0.5 at 2 mL/min as mobile phase and UV detection at 270 nm



Figur 4.5.3. Thymidine phosphorolysis time course by *E. coli* thymidine phosphorylase at 37°C. Thymidine (250 nmole) was incubated with *E. coli* thymidine phosphorylase (0.2 u). The reaction was terminated by the addition of ice-cold methanol. The data shown are means from two replicate determinations.

Table 4.5.1. *In vitro* phosphorolysis of AZT, Thd, d4T, DU, EDU and their 5-halo-6-methoxy (or azido)-5,6-dihydro analogs by *E. coli* thymidine phosphorylase at 37°C. The phosphorolysis was determined at 10 min after the addition of the individual test compound (250 nmole) to *E. coli* thymidine phosphorylase (0.2 u). The reaction was terminated by the addition of ice-cold methanol. The data shown are means from n=3 for AZT and its 5,6-dihydro analogs. n=2 for the rest of the test compounds.

Compound	% Phosphorolysis
Azidothymidine (AZT)	0
cis-(+)-(5S,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTBrOMe	0
trans-(-)-(5S,6S)-AZTBrOMe	0
trans-(+)-(5R,6R)-AZTIOMe	0
trans-(-)-(5S,6S)-AZTIOMe	0
Thymidine (Thd)	72
$ThdBrN_3$[a]	0
2',3'-Dideoxy-2',3'-didehydrothymidine (d4T)	10
$DDTBrN_3$[b]	0
cis-(+)-(5S,6R)-d4TClOMe	0
trans-(+)-(5R,6R)-d4TClOMe	0
trans-(+)-(5R,6R)-d4TBrOMe	0
trans-(-)-(5S,6S)-d4TBrOMe	0
trans-(+)-(5R,6R)-d4TIOMe	0
trans-(-)-(5S,6S)-d4TIOMe	0
2'-Deoxyuridine (DU)	57
$DUBrN_3$[c]	0
Edoxudine (5-ethyl-2'-deoxyuridine, EDU)	12
$EDUClN_3$[d]	0

[a] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-azido-5,6-dihydrothymidine.
[b] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-azido-5,6-dihydro-2',3'-dideoxy-2',3'-didehydrothymidine.
[c] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-azido-5,6-dihydro-2'-deoxyuridine.
[d] Mixture of trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-chloro-6-azido-5,6-dihydro-5-ethyl-2'-deoxyuridine.

4.6. Chemical stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT.

Chemical stability on standing is a prerequisite for any drug. Prodrugs and drugs must be sufficiently stable to have a reasonable shelf-life. However, the determination of shelf-life by accelerated studies is beyond the scope of this thesis. The chemical stability of 5-halo-6-methoxy-5,6-dihydro analogs of AZT, upon incubation with phosphate buffer, was therefore determined. Trans-(+)-(5R,6R)-5-chloro (bromo or iodo)-6-methoxy-5,6-dihydro analogs of AZT were incubated with phosphate buffer (pH 7.0, 0.06 M) at a concentration of 1 mM at 37°C for 24 h. The results, summarized in Table 4.6.1., indicate that all are stable for at least 24 h at 37°C. There was no sign of regeneration of AZT.

Table 4.6.1. The stability of 5,6-dihydro analogs upon incubation with phosphate buffer (pH 7.0, 0.06 M) at a concentration of 1 mM at 37°C.

Compound added	**5R,6R-(+)-AZTClOMe**		**5R,6R-(+)-AZTBrOMe**		**5R,6R-(+)-AZTIOMe**	
Compound measured	**% as prodrug**	**% AZT generated**	**% as prodrug**	**% AZT generated**	**% as prodrug**	**% AZT generated**
Time (h)						
0	99.54	0	99.02	0	95.08	0
1.0	99.33	0	99.11	0	99.75	0
3.0	99.06	0	98.76	0	90.57	0
5.0	99.59	0	99.23	0	98.44	0
10.0	99.34	0	99.07	0	97.36	0
24.0	99.47	0	99.21	0	99.76	0

4.7. *In vitro* regeneration of parent drugs from their 5-halo-6-methoxy-5,6-dihydro analogs.

In vitro regeneration of parent drugs from their 5-halo-6-methoxy-5,6-dihydro analogs was investigated in the presence of glutathione (GSH), mouse whole blood and the soluble enzyme fraction of mouse liver.

4.7.1. Conversion of the trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro analog of AZT to AZT by glutathione.

The usefulness of a prodrug is dependent not only on its partition coefficient, transport characteristics and chemical stability, but also on its conversion to its active form. To be effective, prodrugs must convert to their active drug after administration since prodrugs are generally inactive.

The regeneration of uracils or uracil bases from their 5,6-dihydro analogs through thiol-mediated processes has been reported earlier.[318-323] 5-Bromo-6-methoxy-5,6-dihydrothymidine has been studied as a model compound to determine the mechanisms for its conversion to thymine by cysteine. When regeneration of uracils or uracil bases from their 5,6-dihydro analogs occurs via a thiol-mediated process, two mechanisms (E2 and SN2 as presented in Figure 4.7.1.) for the thiol-mediated dehalogenation are possible.[319] As shown in pathway A (E2 reaction), elimination of the C^5 halo substituent (X) involving a nucleophilic attack by glutathione (GSH) would give a carbanion or enolate anion (ii) as an intermediate. Alternatively, in pathway B (SN2 reaction), displacement of X by GSH to give (iii), followed by the subsequent attack of another molecule of GSH, would give the same intermediate (ii). Then elimination of methoxide anion from (ii) would yield parent nucleobase or nucleoside. However, the relative contributions of these mechanisms likely vary depending upon reactant concentrations, the nature of the halogen atom, and the substituents on the uracil ring system.[319] 5-Bromo-5-fluoro-5,6-dihydro-2'-

deoxyuridine has therefore been reported to act as a slow glutathione-mediated releaser of 5-fluoro-2'-deoxyuridine.[231] More recently, 5-halo-5-ethyl-6-methoxy-5,6-dihydro-2'-deoxyuridines have also been shown to be prodrugs of 5-ethyl-2'-deoxyuridine, with increased brain uptake and blood residence time.[232]

In this study, we attempted to investigate the conversion of the 5,6-dihydro prodrugs to their corresponding active drugs. First, the conversion of trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro analog of AZT to AZT in the presence of varying amounts of glutathione was determined for specified reaction times. The results shown in Table 4.7.1. indicate that the conversion was dependent on the ratio of substrate:glutathione and was almost 100% when the substrate:glutathione molar ratio of 1:2 was used. In all cases the conversion was complete within 30 min.

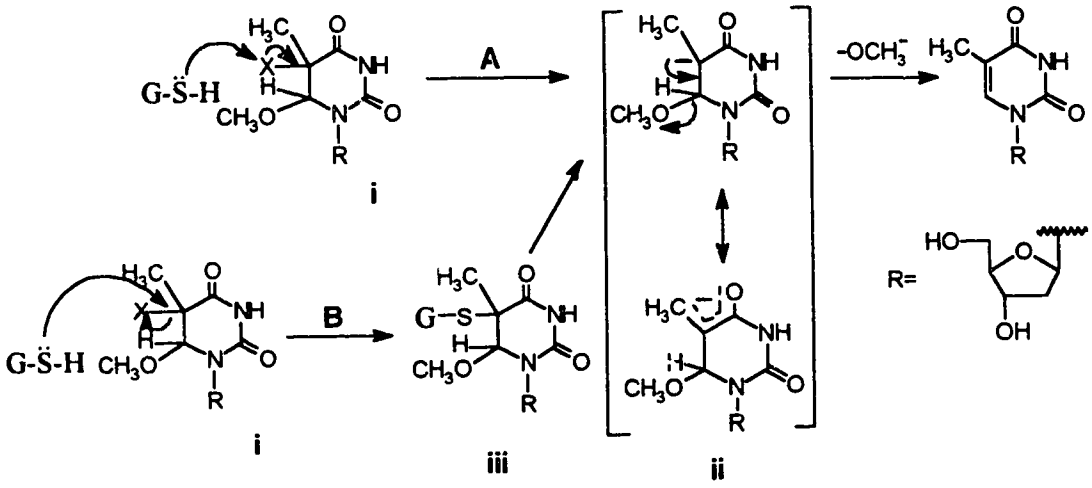


Figure 4.7.1. Putative mechanisms for the regeneration of uracil nucleoside or uracil nucleobase from their 5,6-dihydro analogs.[231]

Table 4.7.1. Regeneration of AZT from its trans-(+)-(5R,6R)-5-bromo-6-methoxy-5,6-dihydro analog by glutathione. Trans-(+)-(5R,6R)-AZTBrOMe was incubated with glutathione at different molar ratios for different time periods at 37°C. The results are from single determination only.

Molar ratio (substrate:glutathione)	**% regeneration of AZT**		
	30 min	60 min	90 min
1:0.2	11	8	9
1:2	98	99	ND[a]
1:10	100	ND[a]	ND[a]

a. ND = not determined.

The results suggest that the reaction is dependent upon the amount of glutathione and that 30 min is a sufficient reaction time for the reaction to go to completion. A substrate:glutathione molar ratio of 1:2, and a reaction time of 30 min was therefore selected for subsequent regeneration of parent drugs from corresponding 5,6-dihydro analogs by glutathione.

4.7.2. Regeneration of AZT, FLT and d4T from their corresponding 5-halo-6-methoxy-5,6-dihydro analogs by glutathione.

The chemical reactivity of glutathione and its potential involvement in the dehalogenation of the 5-halo-6-methoxy-5,6-dihydro analogs gives important insights into the more complicated *in vivo* process because of the presence of glutathione, cysteine and enzymes containing a thiol group.

Glutathione is an important substance with immune-enhancing and antioxidant properties. Extracellular glutathione protects cells against oxidation injury and is a major

transport form of the amino acid cysteine. Within cells, glutathione functions as an antioxidant. Glutathione is also believed to be important in the initiation and progression of lymphocyte activation, and thus essential for host defense. The normal glutathione level in plasma is 5.99 μM, of which 5.71 μM is in the form of reduced glutathione. In mammalian tissues, the glutathione concentration is in the range of 0.5-10 mM, whereas the cysteine concentration is in the range of 0.03-0.1 mM.[352] However, the level of glutathione in HIV seropositive group is about one third of that in normal people.[353]

It was of interest to design prodrugs which would provide optimal regeneration of active drugs in AIDS patients and provide a sustained release of active drug *in vivo*. Based on the mechanisms proposed, the thiol-mediated dehalogenation process is dependent on the nature of the halogen species, the steric effect and electronegativity of different halogens, as demonstrated by Sander *et al*.[322] In their studies, 5-FU was resistant to dehalogenation, while 5-chloro-, 5-bromo- and 5-iodo- uracils underwent dehalogenation. Another study reported by Duschinsky *et al* showed regeneration of the 5,6-double bond for various 5,6-disubstituted 5-fluorodihydrouracils.[231] It was found that 5-chloro-6-alkoxy-5,6-dihydro-5-fluorouridines did not convert to FUDR, while 5-bromo-6-alkoxy-5,6-dihydro-5-fluorouridines converted at different rates. It has also been shown that the % regeneration of FUDR from 5-bromo-6-alkoxy-5,6-dihydro-5-fluorouridines is slightly decreased upon the elongation of the chain at C^6 in the rank of $-OCH_3 > -OC_2H_5 > -OC_3H_7$. In addition, the conversion is dependent on the substituents on the uracil ring system.[231,322]

The regeneration of AZT, FLT and d4T from their 5-halo-6-alkoxy-5,6-dihydro analogs was therefore investigated. The individual test compound was incubated with glutathione using a substrate:glutathione molar ratio of 1:2 at 37°C based on the results in Table 4.7.1. After specified time periods, the sample was analyzed by HPLC. The results are summarized in Tables 4.7.2.-4.7.4. The regeneration of the parent drugs from their 5,6-dihydro analogs were found to be dependent on the nature of the halogen at the C^5

position. In general, both iodo- and bromo- analogs converted to their parent drugs in the presence of glutathione with a relative % regeneration of -I > -Br, while chloro analogs were stable (no conversion was observed). The results suggest that when the halogen at C^5 position is changed from chloro to bromo or iodo, this susceptibility to attack by glutathione is increased dramatically due to the fact that bromine and iodine are better leaving groups than chlorine. Conversion of 5-halo-6-alkoxy-5,6-dihydro analogs that have alkoxy groups other than the methoxy group was not studied. Based on the mechanisms proposed in Figure 4.7.1., the difference in conversion rate from two trans-diastereomers [trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-] should not be significant because of the similar electronic and steric effects for both isomers. However, the difference between cis- and trans- isomers is dependent on which mechanism is more involved. If the E2 mechanism plays a more important role, the conversion from the cis-isomer should be slower than from the trans- isomer, because it presents a bigger steric hindrance to the approach of the nucleophile than trans- isomer does. In contrast, if SN2 is more involved, the conversion from the trans- isomer will be slower than that from the cis- isomer, because the attack of the nucleophile is from the other side of the halogen and the cis- isomer would exhibit a smaller steric hindrance relative to the trans- isomer. However, a large difference was observed between the trans-(-)-(5S,6S)-FLTIOMe (90%) and the trans-(+)-(5R,6R)-FLTIOMe (50%) isomers. Trans-(-)-(5S,6S)-d4TBrOMe (50%) and trans-(+)-(5R,6R)-d4TBrOMe (85%) also showed different % regenerations.

Table 4.7.2. Regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs by glutathione. The individual test compound was incubated with glutathione at a molar ratio (substrate:glutathione) of 1:2 for specified time period at 37°C. The results are means of two determinations.

Substrate	Molar ratio (substrate:glutathione)	Incubation time	% Conversion
trans-(+)-(5R,6R)-AZTIOMe	1:2	30 min	100
trans-(-)-(5S,6S)-AZTIOMe	1:2	30 min	100
trans-(+)-(5R,6R)-AZTBrOMe	1:2	30 min	98
trans-(-)-(5S,6S)-AZTBrOMe	1:2	30 min	90
cis-(+)-(5S,6R)-AZTClOMe	1:2	24 h	0
trans-(+)-(5R,6R)-AZTClOMe	1:2	24 h	0

Table 4.7.3. Regeneration of FLT from its 5-halo-6-methoxy-5,6-dihydro analogs by glutathione.[315] The individual test compound was incubated with glutathione at a molar ratio (substrate:glutathione) of 1:2 for specified time period at 37°C. The results are means of two determinations.

Substrate	Molar ratio (substrate:glutathione)	Incubation time	% Conversion
trans-(+)-(5R,6R)-FLTIOMe	1:2	30 min	50
trans-(-)-(5S,6S)-FLTIOMe	1:2	30 min	90
trans-(+)-(5R,6R)-FLTBrOMe	1:2	30 min	10
trans-(-)-(5S,6S)-FLTBrOMe	1:2	30 min	10
cis-(+)-(5S,6R)-FLTClOMe	1:2	30 min	0
trans-(+)-(5R,6R)-FLTClOMe	1:2	30 min	0

Table 4.7.4. Regeneration of d4T from its 5-halo-6-methoxy-5,6-dihydro analogs by glutathione. The individual test compound was incubated with glutathione at a molar ratio (substrate:glutathione) of 1:2 for specified time period at 37°C. The results are means of two determinations.

Substrate	Molar ratio (substrate:glutathione)	Incubation time	% Conversion
trans-(+)-(5R,6R)-d4TIOMe	1:2	30 min	>95
trans-(-)-(5S,6S)-d4TIOMe	1:2	30 min	>95
trans-(+)-(5R,6R)-d4TBrOMe	1:2	30 min	85
trans-(-)-(5S,6S)-d4TBrOMe	1:2	30 min	50
cis-(+)-(5S,6R)-d4TClOMe	1:2	21 h	0
trans-(+)-(5R,6R)-d4TClOMe	1:2	21 h	0

Compared to 5,6-dihydro-5-fluorouridines[231] such as the 5-bromo-6-alkoxy analogs which showed only 18-21% conversion to the parent compound after 24 h, the 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs studied in this thesis convert to their parent compound much more completely (50-100% after 30 min). The results support previous findings that the conversion from 5,6-dihydro analogs is dependent on the specific substituents on the uracil ring. The % regeneration of parent nucleosides from their corresponding 5,6-dihydro analogs was AZT > d4T > FLT.

4.7.3. Regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in mouse blood.

The results from the glutathione study indicated that the regeneration of AZT, FLT or d4T from their 5-halo-6-methoxy-5,6-dihydro analogs by glutathione was dependent upon the nature of the halogen atom, being easily regenerated from their 5-iodo (or

bromo) analogs, but not from the chloro analogs. A similar relationship is postulated to occur in blood due to the presence of GSH, cysteine or enzymes containing a thiol moiety. It has been shown that the 5-bromo-6-methoxy-5-ethyl-5,6-dihydro analog of EDU is converted to EDU in both blood and plasma, and that the conversion in blood (58%) was more extensive than in plasma (8%), suggesting that blood cells play an important role in this conversion.[232]

In vitro studies of 5-halo-6-methoxy-5,6-dihydro analogs were conducted using mouse whole blood. The regeneration of AZT *in vitro* in mouse blood more closely represents the biological environment the prodrugs will experience than incubation with GSH. The advantage of the *in vitro* mouse blood study over an *in vivo* study is that other systemic effects which would complicate analysis of the data can be avoided.

Regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in mouse blood was investigated by incubating 100 μL of heparinized Balb/c mouse blood with 100 μg of the individual test compound for 10 min. The sample was then analyzed by HPLC. The results, presented in Table 4.7.5., suggest that regeneration of AZT is again dependent upon the nature of the halogen atom at the C^5 position, where the % regeneration order is I > Br > Cl. After a 10 min incubation, 5-iodo-6-methoxy-5,6-dihydro analogs of AZT were completely converted and 5-bromo-6-methoxy-5,6-dihydro analogs were partially converted to AZT. Furthermore, 5-chloro-6-methoxy-5,6-dihydro analogs were not converted at all. The trans-(+)-(5R,6R)- and trans-(-)-(5S,6S)-5-bromo-6-methoxy-5,6-dihydro analogs of AZT showed slightly different amounts of conversion.

Table 4.7.5. The regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in mouse blood. The individual test compound was incubated with mouse whole blood for 10 min at 37°C. The data are from single determination only.

Compound	Regeneration %
trans-(+)-(5R,6R)-AZTIOMe	100
trans-(-)-(5S,6S)-AZTIOMe	100
trans-(+)-(5R,6R)-AZTBrOMe	59
trans-(-)-(5S,6S)-AZTBrOMe	88
cis-(+)-(5S,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTClOMe	0

4.7.4. Regeneration of AZT and d4T from their 5-halo-6-methoxy-5,6-dihydro analogs in the soluble enzyme fraction of mouse liver.

Liver is rich in enzymes and is an important site of drug metabolism. The regeneration of AZT and d4T from their 5-halo-6-methoxy-5,6-dihydro analogs were examined by incubation of the test compound with a soluble liver enzyme fraction for 30 min. The sample was then analyzed by HPLC. The results are listed in Tables 4.7.6 and 4.7.7.

Again it was found that the regeneration of AZT or d4T from their 5,6-dihydro analogs is dependent on the nature of the halogen at C^5 and that 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs converted to their corresponding nucleoside analogs while 5-chloro-6-methoxy-5,6-dihydro analogs did not convert under the same conditions.

Table 4.7.6. The regeneration of AZT from its 5-halo-6-methoxy-5,6-dihydro analogs in a soluble enzyme fraction of mouse liver. The individual test compound was incubated with the soluble enzyme fraction of mouse liver for 30 min at 37°C. The data shown are from single determination only.

Compound	Regeneration%
trans-(+)-(5R,6R)-AZTIOMe	80
trans-(-)-(5S,6S)-AZTIOMe	95
trans-(+)-(5R,6R)-AZTBrOMe	26
trans-(-)-(5S,6S)-AZTBrOMe	43
cis-(+)-(5S,6R)-AZTClOMe	0
trans-(+)-(5R,6R)-AZTClOMe	0

Table 4.7.7. The regeneration of d4T from its 5-halo-6-methoxy-5,6-dihydro analogs in a soluble enzyme fraction of mouse liver. The individual test compound was incubated with the soluble enzyme fraction of mouse liver for 30 min at 37°C. The data shown are from single determination only.

Compound	Regeneration%
trans-(+)-(5R,6R)-d4TIOMe	85
trans-(-)-(5S,6S)-d4TIOMe	95
trans-(+)-(5R,6R)-d4TBrOMe	75
trans-(-)-(5S,6S)-d4TBrOMe	52
cis-(+)-(5S,6R)-d4TClOMe	0
trans-(+)-(5R,6R)-d4TClOMe	0

4.8. Blood levels of AZT in mice obtained after i.v. injection of AZT or its 5-halo-6-methoxy-5,6-dihydro analogs.

An *in vivo* study using Balb/c mice was conducted by i.v injection of AZT and its 5-halo-6-methoxy-5,6-dihydro analogs (144 μmol/kg) via the tail vein. The concentrations of AZT and the individual 5,6-dihydro analog in plasma were determined by HPLC analysis after extraction by Sep-Pak cartridges. However, the presence of the 5,6-dihydro analogs after administration of the 5-bromo and 5-iodo-6-methoxy-5,6-dihydro analogs was not detectable, which is due to the limited sensitivity (approximately 1μg/mL) of HPLC-UV detection and their fast regeneration to AZT. AZT was therefore quantitated, using FIAU as an internal standard. The recovery of AZT and FIAU from the Sep-Pak was 88±5% and 84±4%, respectively. In the case of the 5-chloro-6-methoxy-5,6-dihydro analog of AZT (AZTClOMe), quantitation of either AZT or AZTClOMe after i.v. injection of AZTClOMe was problematic due to the low quantity of AZT generated from AZTClOMe, and the low UV absorbance of AZTClOMe. The dose of trans-(+)-(5R,6R)-AZTClOMe was therefore increased to 240 μmol/kg. Both AZT and AZTClOMe were then detected using FLTBrOMe as an internal standard. The blood levels of AZT in Balb/c mice after i.v. injection of AZT, trans-(+)-(5R,6R)-AZTBrOMe, trans-(-)-(5S,6S)-AZTBrOMe, trans-(+)-5R,6R)-AZTIOMe or trans-(-)-(5S,6S)-AZTIOMe (144 μmol/kg) are presented in Table 4.8.1. and Figure 4.8.1. These results demonstrate that the level of AZT in blood is similar after injection of the same molar dose of either AZT or its 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs. The concentrations of both AZT and trans-(+)-AZTClOMe after administration of trans-(+)-AZTClOMe are shown in Table 4.8.2.

The *in vivo* results suggest that both AZTBrOMe and AZTIOMe are rapidly converted to AZT, thereby providing similar blood levels of AZT as those observed after

Table 4.8.1. Blood levels of AZT after i.v. administration of AZT and its 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs (144 μmol/kg). The data shown are means ± SD, n=3.

Compound administered	AZT	5R,6R-(+)-AZTBrOMe	5S,6S-(-)-AZTBrOMe	5R,6R-(+)-AZTIOMe	5S,6S-(-)-AZTIOMe
Compound measured	AZT (μg/mL)	AZT (μg/mL)	AZT (μg/mL)	AZT (μg/mL)	AZT (μg/mL)
Time (min)					
5	37.50±5.87	20.9±2.4	37.67±5.90	38.00±5.01	35.80±7.23
15	23.37±8.31	9.51±1.27	21.63±3.46	16.06±7.60	11.50±1.32
30	8.72±0.72	6.70±1.15	10.98±6.08	11.70±5.00	7.77±1.16
45	6.38±1.83	2.70±0.67	9.40±6.77	5.75±0.90	ND
60	2.85±1.12	2.00±0.89	2.20±0.66	3.60±1.64	0.65±0.07
90	NDT	1.30±0.56	1.23±0.53	1.65±0.40	2.00±0.00
120		NDT	0.53±0.08	1.23±0.49	1.80±0.85
150			NDT	NDT	1.50±0.26

ND = not determined.

NDT = not detectable.

AZT administration. However the % regeneration of AZT from AZTClOMe was very low, resulting in a relatively low concentration of AZT (less than 1/10 of that obtained from the 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs).

This study again demonstrated the impact of the halogen at C^5 in the 5,6-dihydro analogs on the regeneration of AZT. Both 5-bromo and iodo analogs resulted in a similar % regeneration of AZT, which was much larger than for the 5-chloro analog.

A compound with a moderate % regeneration is needed to provide a sustained release of AZT. Based on the two mechanisms proposed (shown in Figure 4.7.1, page 109) and previous reports that the dehalogenation processes were dependent on both substituents at C^5 and C^6 positions,[231-233,318-323] it is postulated that alkoxy moiety at C^6 in 5,6-dihydro analogs of AZT should have an impact on the rate and %conversion of 5,6-dihydro analogs of AZT to AZT in addition to the impact observed from the halogen

Table 4.8.2. Analysis results of blood samples after i.v. injection of trans-(+)-(5R,6R)-5-chloro-6-methoxy-5,6-dihydro analog of AZT (240 μmol/kg). The data shown are means from n=2.

Time	15 min	30 min	60 min	90 min
AZT concentration (μg/mL blood)	2.5	2.5	1.3	NDT
AZTClOMe concentration (μg/mL blood)	28.8	12.5	16.3	1.1

NDT = not detectable.

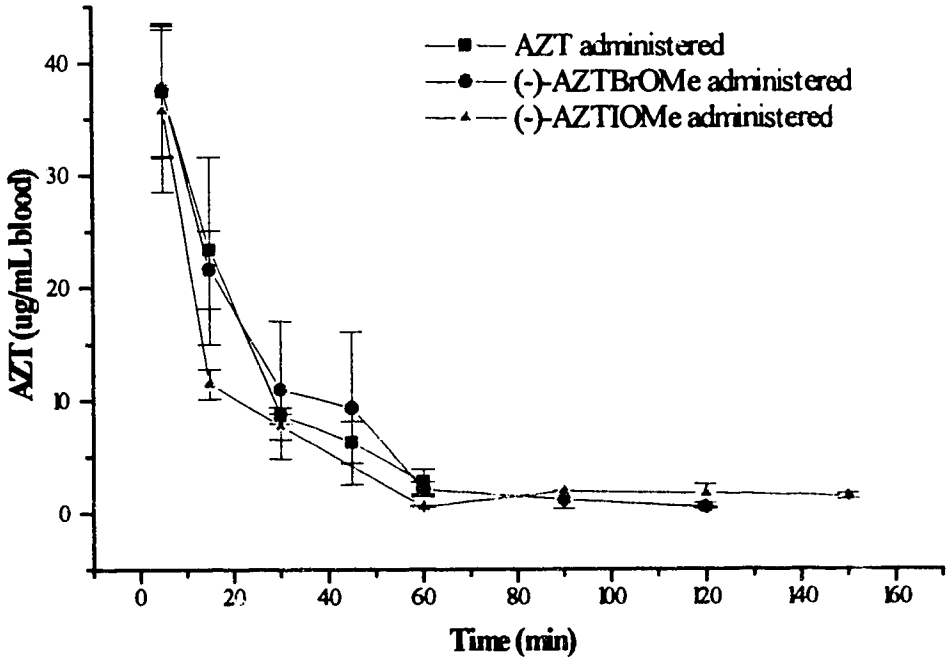


Figure 4.8.1. The blood levels of AZT obtained after i.v. injection of AZT (144 μmol/kg) and its trans-(-)-(5S,6S)-5-bromo(or iodo)-6-methoxy-5,6-dihydro analogs (equimolar concentration), n=3.

at the C^5 position. Therefore, a 5-halo-6-alkoxy-5,6-dihydro analog of AZT with a moderate conversion rate and % conversion to AZT may be designed by an appropriate combination of substituents at C^5 and C^6. The study involving compounds that possess different alkoxy groups at the C^6 position is under investigation by others in our research group. However, this study was done in normal mice. The regeneration of AZT from the 5-halo-6-alkoxy-5,6-dihydro analogs may be a rate-limiting process in infected patients if the regeneration of AZT is a thiol-mediated process since the glutathione level in HIV infected subjects is lower than in uninfected individuals. A dosage of 200 mg (0.75 mmole) of AZT every 8 h is now more commonly used. If an equimolar concentration of the 5,6-dihydro analog is given, 1.5 mmole glutathione is needed to convert all of the prodrug (5,6-dihydro analog) molecules to AZT. However glutathione (5.99 μM in plasma) is not the only substance that exists in the body that is involved in thiol-mediated reactions. For example, cysteine (0.03-0.1 mM in mammalian tissues) and enzymes containing thiol moiety are also available for this regeneration.

As discussed earlier, many ester prodrugs of AZT have been proposed, including DP-AZT and 5'-aliphalic acid-AZTs. Although DP-AZT was designed to target in brain, the concentration of AZT in plasma is constantly lower (50%) than that achieved after AZT itself due to the fast clearance of DP-AZT from blood. 5'-Aliphatic acid-AZTs have been the most promising in producing a sustained release of AZT in plasma to date.[205] AZT concentrations in plasma after 5'-aliphatic acid-AZTs by intraperitoneal administration were shown to be more constant and persistent, especially when the acyl chain is increased to C18 as a result of longer retention due to its significantly increased lipophilicity and sustained enzymatic hydrolysis.

4.9. Biodistribution for [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in mice.

Our primary objective is to develop prodrugs of nucleoside RT inhibitors that will have high brain uptake. The biodistribution studies in mice were performed to determine if the enhanced lipophilicity of the 5,6-dihydro analogs resulted in an increased brain uptake.

The *in vivo* distribution study of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe, [2-^{14}C]-trans-(-)-(5S,6S)-AZTBrOMe and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt after i.v. injection via the tail vein was carried out in conventional Balb/c mice. The amount of radioactivity in blood, brain, bone, heart, liver, spleen, lung, kidney, bladder and urine at several time intervals was determined using a combustion/liquid scintillation counting method. The combustion efficiency ranged between 84-97%. The results presented in Tables 4.9.1.- 4.9.4. are percentage of the administered radioactivity recovered per g or per mL of tissue, i.e., ID%/g or mL tissue (ID=injected dose). The results for urine samples were based on the small volume of urine excreted, calculated per mL urine sample. The number greater than 100% is due to the fact that a small volume (< 1 mL) was actually excreted. The data suggest that the test compounds (both AZT and its 5,6-dihydro analogs) are distributed to all of the tissues examined. The extensive distribution of the test compounds into various tissues, particularly at very early time periods, is likely due to their high lipophilicity. The high level of radioactivity in kidney, bladder and urine suggests that renal clearance is a major excretion pathway for these compounds.

These experiments have provided very exciting findings which include an increased concentration in brain and prolonged blood levels, together with no increase in bone after injection of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe, [2-^{14}C]-trans-(5S,6S)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt as compared to an equimolar dose

of [2-^{14}C]-AZT. Although AZT has been approved for the treatment of AIDS since 1987, its bone marrow toxicity, limited brain uptake and poor blood kinetic characteristics have challenged both clinicians and researchers involved in the treatment of AIDS patients and in the development of more efficient anti-AIDS compounds, respectively. To reduce bone marrow toxicity, lower doses of AZT have been recommended,[354] but this may result in lower brain levels, thereby decreasing AZT's therapeutic effectiveness for children and for cerebral infection.[19] Many types of prodrugs have been made in an attempt to overcome this problem. To date, all efforts have resulted in failure to produce a prodrug with a clear-cut therapeutic advantage. Among the prodrugs reported in the literature, the DP-AZT appears to be the most successful, producing significant increases in brain delivery in animal studies.[257] DP-AZT resulted in a three-fold-higher concentration of AZT in rat brain, and a two-fold increase in concentrations in the CSF was observed in dog studies.[257] However, the biodistribution of DP-AZT was not examined. Since a lower concentration of AZT is found in plasma after DP-AZT dosing, it is therefore not known if DP-AZT will provide a clinical advantage. The 5,6-dihydro analogs investigated in this study provided a three-fold increase in brain uptake in mice, which is similar to the result obtained from DP-AZT.

Although the *in vivo* conversion of the 5-bromo (or 5-iodo)-6-methoxy-5,6-dihydro analogs to the parent compound was very fast, as shown in Section 4.8., no significant differences in blood concentrations of AZT after administration of AZT or its 5-bromo (or 5-iodo)-6-methoxy-5,6-dihydro analogs, was observed within 2 h post injection. The biodistribution study did show an improvement with respect to brain targeting and in blood at longer time periods after 2 h (Tables 4.9.1.-4.9.4.).

Table 4.9.1. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-AZT (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3

Tissue	10 min	30 min	60 min	120 min	240 min	360 min
Blood[1]	4.15±0.21	1.48±0.29	0.54±0.13	0.09±0.03	0.03±0.03	0.03±0.02
Heart[2]	4.43±0.34	1.52±0.35	0.65±0.16	0.13±0.03	0.02±0.01	Trace
Liver[2]	5.31±0.58	1.81±0.38	0.73±0.15	0.18±0.05	0.06±0.03	Trace
Spleen[2]	4.94±0.02	2.15±0.83	1.07±0.21	0.54±0.17	0.09±0.07	0.05±0.03
Lung[2]	5.01±0.52	1.74±0.33	0.67±0.17	0.12±0.05	0.03±0.01	Trace
Bone[2]	3.48±0.23	1.48±0.47	0.67±0.13	0.27±0.06	0.28±0.24	0.03±0.01
Brain[2]	0.35±0.03	0.16±0.04	0.10±0.02	0.04±0.01	0.03±0.01	Trace
Kidney[2]	14.7±44.0	4.14±0.65	3.35±1.63	0.31±0.20	0.03±0.01	Trace
Bladder[2]	17.50±4.14	11.19±5.73	14.09±12.17	5.53±8.60	8.33±10.29	Trace
Urine[1]	312±98	1203±654	877±338	259±352	62±33	5.61±6.60

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g of tissue.

Table 4.9.2. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-trans-(-)-(5S,6S)-AZTBrOMe (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Tissue	10 min	30 min	60 min	90 min	120 min	210 min	360 min	480 min
Blood[1]	1.05±0.03	1.05±0.15	0.90±0.15	0.91±0.20	0.75±0.17	0.75±0.04	0.93±0.04	0.84±0.19
Heart[2]	4.24±0.58	1.08±0.16	0.71±0.15	0.43±0.06	0.29±0.07	0.23±0.08	0.34±0.02	0.28±0.02
Liver[2]	5.79±0.62	1.23±0.38	0.71±0.24	0.60±0.25	0.23±0.01	0.23±0.02	0.25±0.07	0.19±0.03
Spleen[2]	5.16±1.23	1.24±0.36	1.22±0.28	1.05±0.41	0.42±0.03	0.30±0.07	0.30±0.02	0.28±0.04
Lung[2]	4.71±1.15	1.16±0.29	0.74±0.14	0.45±0.20	0.37±0.09	0.37±0.04	0.29±0.13	0.32±0.03
Bone[2]	3.24±0.24	1.83±0.35	0.49±0.11	0.40±0.12	0.34±0.19	0.13±0.01	0.11±0.01	0.11±0.01
Brain[2]	0.69±0.17	0.30±0.12	0.15±0.01	0.13±0.04	0.12±0.04	0.02±0.01	0.05±0.01	0.05±0.02
Kidney[2]	16.97±4.16	1.97±1.03	1.11±0.18	0.56±0.06	0.40±0.02	0.21±0.03	0.22±0.02	0.20±0.02
Bladder[2]	29.14±11.12	41.15±30.39	11.49±8.53	8.62±7.78	1.95	0.61±0.32	0.27±0.02	0.27±0.04
Urine[1]	292±158	ND	259±113	268±84	ND	9.27±4.44	3.17±1.11	0.94±0.04

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g of tissue.

ND = not determined.

Table 4.9.3. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Tissue	10 min	30 min	60 min	120 min	240 min	360 min
Blood[1]	3.42±0.51	1.48±0.32	1.32±0.17	0.77±0.15	0.88±0.07	0.67±0.16
Heart[2]	3.22±0.48	1.45±0.19	0.76±0.06	0.38±0.05	0.27±0.03	0.21±0.05
Liver[2]	4.17±0.50	1.49±0.34	0.83±0.20	0.23±0.02	0.17±0.02	0.16±0.05
Spleen[2]	3.43±0.35	1.95±0.69	1.59±0.60	0.44±0.04	0.[illegible]	0.18±0.06
Lung[2]	3.64±0.39	1.57±0.10	1.09±0.56	0.38±0.01	0.[illegible]	0.25±0.06
Bone[2]	2.32±0.57	1.01±0.16	0.89±0.19	0.20±0.02	0.10±0.01	0.06±0.01
Brain[2]	1.06±0.18	0.48±0.08	0.38±0.23	0.11±0.01	0.07±0.01	0.04±0.01
Kidney[2]	10.04±1.19	4.56±1.26	1.94±0.58	0.31±0.05	0.18±0.01	0.13±0.03
Bladder[2]	25.70±11.82	64.66±19.76	68.05±51.16	4.33±2.66	0.50±0.27	0.34±0.22
Urine[1]	818±877	835±93	800±432	55±16	8.2±2.05	8.38±6.85

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g of tissue.

Table 4.9.4. Distribution of radioactivity in mice after tail vein injection of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Tissue	10 min	30 min	60 min	120 min	240 min	360 min	480 min
Blood[1]	4.14±0.86	2.20±0.89	0.98±0.25	0.76±0.05	0.64±0.09	0.60±0.07	0.55±0.18
Heart[2]	4.21±0.30	1.64±0.64	0.52±0.03	0.29±0.01	0.18±0.18	0.17±0.03	0.15±0.04
Liver[2]	8.53±2.64	2.47±0.97	0.85±0.09	0.45±0.06	0.2[illegible]±0.06	0.20±0.02	0.19±0.06
Spleen[2]	4.97±0.45	1.98±0.98	0.85±0.13	0.91±0.13	0.30±0.03	0.19±0.01	0.17±0.04
Lung[2]	4.85±0.29	1.94±0.72	0.71±0.05	0.40±0.02	0.28±0.15	0.24±0.02	0.21±0.07
Bone[2]	2.78±0.94	1.79±1.14	0.66±0.24	0.35±0.01	0.16±0.02	0.09±0.01	0.08±0.02
Brain[2]	1.14±0.12	0.41±0.12	0.21±0.02	0.16±0.03	0.10±0.01	0.06±0.01	0.05±0.01
Kidney[2]	15.18±1.23	4.78±1.47	1.39±0.25	0.43±0.02	0.62±0.15	0.18±0.02	0.17±0.05
Bladder[2]	63.74±41.26	32.40±12.61	24.42±24.82	11.78±11.10	0.32±0.03	Trace	Trace
Urine[1]	774±376	467±47	359±170	462±186	45.31±14.35	1.84±0.81	11.98±8.20

[1] The number is the percentage of the administered radioactivity recovered per mL of sample.

[2] The number is the percentage of the administered radioactivity recovered per g tissue.

4.9.1. Comparison of blood radioactivity levels in mice after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt.

Since the antiviral activity is believed to be due to AZT-triphosphate (AZT-TP), an adequate concentration of AZT-TP should be maintained to achieve the anticipated anti-AIDS effect. Although intracellular AZT-TP has a half-life of 3 to 4 h, the half-life of AZT is only about 1 h and the correlation between intracellular AZT-TP and AZT is not clear.[122] The current therapy using AZT requires frequent dosing. On the other hand, to avoid the undesirable side effects, especially dose-limiting bone marrow toxicity, lower concentrations are preferred.[118] The occurrence of severe anemia and neutropenia in patients receiving a dose of 1500 mg/day is significantly higher than that with 500 mg/day (29% vs. 5%). Another study showed that AZT was discontinued earlier in more subjects in standard treatment (250 mg every 4 hour) than in a lower dose (100 mg every 4 h) group (40% vs. 29%).[354] The same study also demonstrated that the lower dose is as effective as the standard dose. To balance the two factors (adequate concentration for efficacy and lower concentration for reduced toxicity), the prodrug approach has become one of the most researched methods. Prodrugs which have a longer residence time in body and provide a sustained release of the parent drug at concentrations adequate for its anti-HIV activity, but not causing toxicity, are needed. There have been extensive efforts to reduce AZT's clearance from blood.[205-207] The majority of the prodrugs of nucleoside RT inhibitors investigated todate are their 5'-derivatives. Among them, 5'-aliphatic acid-AZTs showed promising results in mice.[205] Caprate and stearate AZT analogs in this series demonstrated a sustained release in plasma. However, more studies are needed for further evaluation. The compounds (5'-aliphatic acid esters) showed a dramatic increase in lipophilicity and have log P values up to greater than 5 in comparison with AZT (log P = −0.31) in a chloroform:phosphate buffer system. As was discussed earlier, a compound with excessive lipid solubility will no longer circulate in the bloodstream, and therefore can

not exhibit its biological activity. The biodistribution of these previously unknown 5,6-dihydro compounds has obviously not been reported.

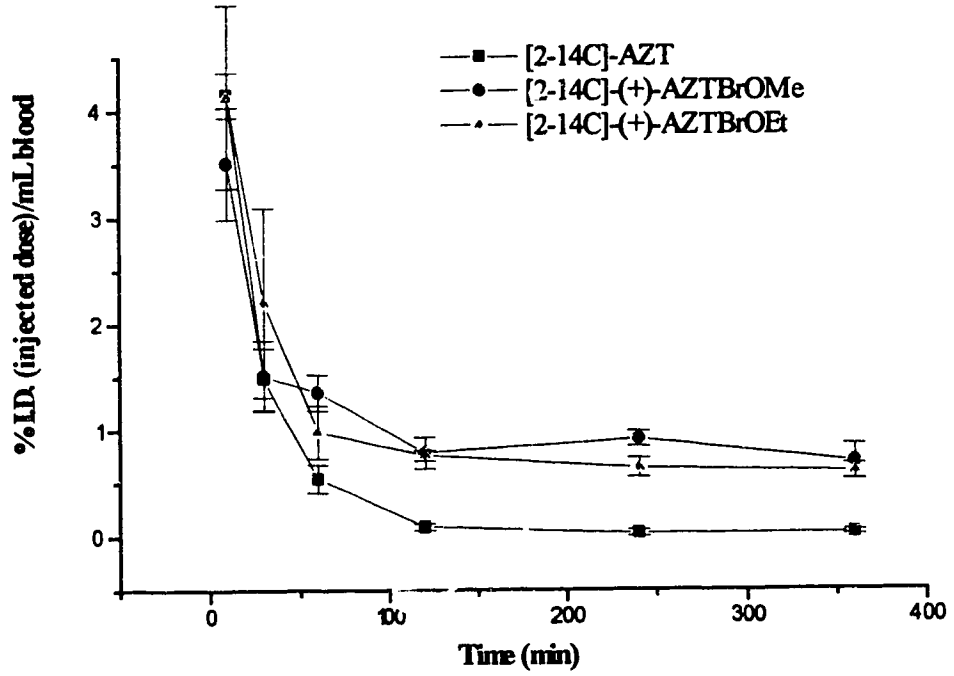


Figure 4.9.1. Clearance of radioactivity from blood after i.v. injection of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs of AZT (2 μCi, specific activity 56 mCi/mmole). Data shown are means ± SD, n=3.

Radioactivity determined in blood up to 6 h after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt expressed as % I.D./mL blood is plotted as shown in Figure 4.9.1. It was found that radioactivity levels from the 5,6-dihydro analogs were significantly higher than those from AZT after 2 h (student T test, $P \leq 0.05$), suggesting that the decline of radioactivity in blood after dosing with the 5-bromo-6-alkoxy (methoxy or ethoxy)-5,6-dihydro analogs is

slower than that of AZT and the difference between blood levels of radioactivity after doses of trans-(+)-(5R,6R)-AZTBrOMe and trans-(+)-(5R,6R)-AZTBrOEt is not significant. The AUCs (area under the curve) are calculated by a traperoidal method and presented in Table 4.9.5. Trans-(+)-(5R,6R)-AZTBrOMe and trans-(+)-(5R,6R)-AZTBrOEt have exhibited significantly increased AUCs relative to that after AZT (346 and 322 vs. 116). In this experiment, only total ratioactivity was measured and the chemical species involved is not completely known. However, based on previous findings regarding the metabolism of AZT, and the results described in this thesis, possible chemical species associated with the radioactivity measured are shown in Figure 4.9.2. The results obtained from this study show that 5,6-dihydro analogs have an increased retention time in the body. The slower clearance of the 5,6-dihydro analogs from the body is likely due to their increased lipophilicity. Greater retention is expected for 5,6-dihydro analogs that have alkoxy groups larger than ethoxy at C^6, because the longer chain at C^6 will likely increase lipophilicity and these analogs should undergo conversion to AZT more slowly.

Table 4.9.5. The AUCs of radioactivity in blood after i.v. injection of ^{14}C-AZT, ^{14}C-trans-(+)-(5R,6R)-AZTBrOMe and ^{14}C-trans-(+)-(5R,6R)-AZTBrOEt in mice.

Compound administered	**[2-^{14}C]-AZT**	**[2-^{14}C]-(5R,6R)-AZTBrOMe**	**[2-^{14}C]-(5R,6R)-AZTBrOEt**
$AUC_{10\text{-}360\ min}$(%ID·min/g)[a]	116	346	322

[a] AUC: Area under the curve which represents the percentage of the administered dose per mL of blood versus time.

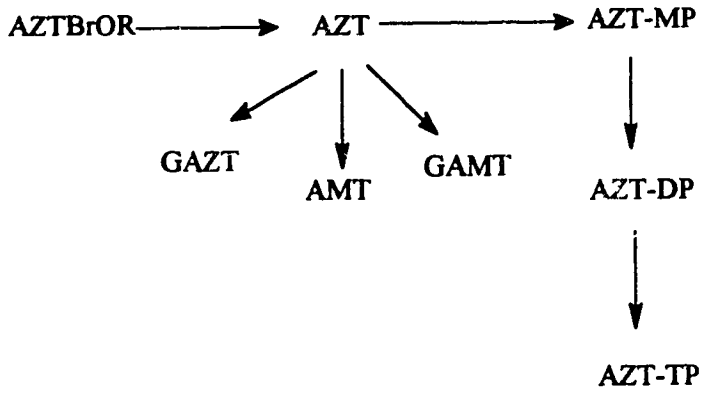


Figure 4.9.2. The postulated metabolic pathways for AZTBrOR.

4.9.2. Comparison of brain radioactivity levels in mice after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt.

Neurological disorders are common in AIDS patients and these disorders contribute to the morbidity of patients in advanced stages of infection with HIV. Isolation of the HIV virus from cerebrospinal fluid and brain tissue has demonstrated the presence of HIV infection in the CNS.[41,124,130-132] Although administration of AZT improves the CNS dysfunction clinically, the penetration of AZT into the brain is believed to be minimal, as demonstrated in rat studies.[134,135] Brain/serum concentration ratios for AZT in mice after administration of 50 mg/kg, and 250 mg/kg, have been reported to be dose dependent, the ratios being higher after doses of 250 mg/kg (brain/serum = 0.283) than those after doses of 50 mg/kg (brain/serum = 0.064). However, due to AZT's dose-related bone marrow toxicity, it is not recommended to increase brain uptake by increasing the administered dose. Moreover, it has been shown that bulk efflux of AZT from CSF and brain via the organic anion transport system plays an important role in the observed limited AZT levels in CSF and brain. These findings underscore the need for antiviral agents, including prodrugs, that can more effectively penetrate the BBB and provide

elevated concentrations of AZT-TP. Various prodrugs of AZT have therefore been designed.

The novel compounds studied in this thesis were designed with the objective of increasing lipophilicity. AZT has been reported to cross cell membranes by passive diffusion although the organic anion transport system mediates the efflux of AZT from CSF and brain and it is known that passive diffusion is increased when lipophilicity is increased. As was discussed earlier, log P's between 0.9 and 2.5 are assumed to be the optimal range for certain radiopharmaceuticals.[355] The 5,6-dihydro analogs of AZT discussed in this thesis have log P values in this range; the exception is cis-(+)-(5S,6R)-AZTClOMe, which has a log P of about 0.5. Therefore a higher delivery to the brain was expected. Indeed, analysis of mouse brain samples showed that the brain radioactivity levels after [2-^{14}C]-5-bromo-6-alkoxy (methoxy or ethoxy)-5,6-dihydro-3'-azido-3'-deoxythymidines (2 μCi, specific activity 56 mCi/mmole) were significantly higher than those after AZT (2 μCi, specific activity 56 mCi/mmole) ($P \leq 0.05$, Figure 4.9.3). At 10 min after i.v. injection, the radioactivity level in brain after administration of the 5,6-dihydro analogs of AZT was three times greater than that after AZT. The ratio of brain/blood radioactivity at 10 min after administration of [2-^{14}C]-AZT was found to be about 0.08. The ratio increased to the 0.28 - 0.65 range for the 5,6-dihydro analogs, suggesting that the 5,6-dihydro analogs have a higher brain uptake due to their increased lipophilicity. Over the range of time periods studied (10 min to 8 h), the radioactivity in brain declined. This is likely due to bi-directional diffusion of AZT regenerated from the prodrug. The AUCs and MRTs (mean residence times) are summarized in Table 4.9.6. The AUCs of the dihydro analogs studied were significantly larger than that for AZT (approximately three-fold). The MRT for the 5-bromo-6-ethoxy-5,6-dihydro analog was significantly increased, although the MRT for the 5-bromo-6-methoxy-5,6-dihydro analog is almost same as that for AZT. This is likely due to a further increase in brain uptake of the 6-ethoxy analog, or slower conversion to AZT. Again only total radioactivity was

determined and the chemical species are not completely understood. The results obtained can be explained by the model depicted in Figure 4.9.4. The prodrug (5,6-dihydro analog) of AZT enters brain more readily because of its enhanced lipophilicity relative to AZT, although regeneration of AZT takes place at the same time. After the prodrug is inside the brain, it can be gradually converted to AZT followed by the formation of AZT-MP, AZT-DP and AZT-TP which are then trapped in the brain because of their greater hydrophilicity. AZT generated either outside or inside the brain will be subject to efflux via the organic anion transport system out of the brain.

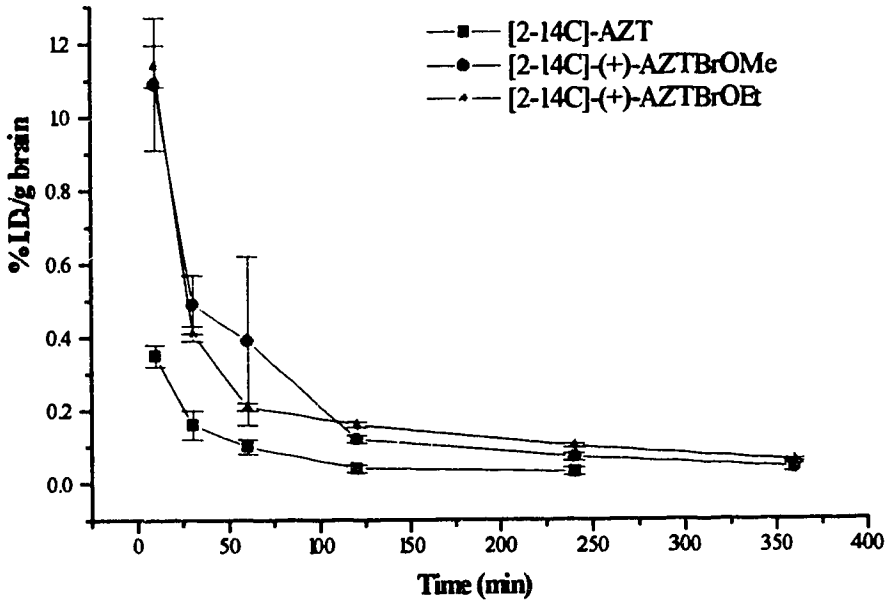


Figure 4.9.3. Brain levels of radioactivity after i.v. injection of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro-3'-azido-3'-deoxythymidines (2 μCi, specific activity 56 mCi/mmole. The data shown are means ± SD. n=3.

Table. 4.9.6. Comparison of radiopharmacokinetic parameters.

Compound administered	[2-^{14}C]-AZT	[2-^{14}C]-Trans-(+)-(5R,6R)-AZTBrOMe	[2-^{14}C]-Trans-(+)-(5R,6R)-AZTBrOEt
$AUC_{0-\infty}$ (%ID·min/g)[a]	17.4	53.8	51.5
Mean residence time (min)	131.5	141.2	236.3

[a]AUC: Area under the curve which represents the percentage of the administered dose per g of brain versus time.

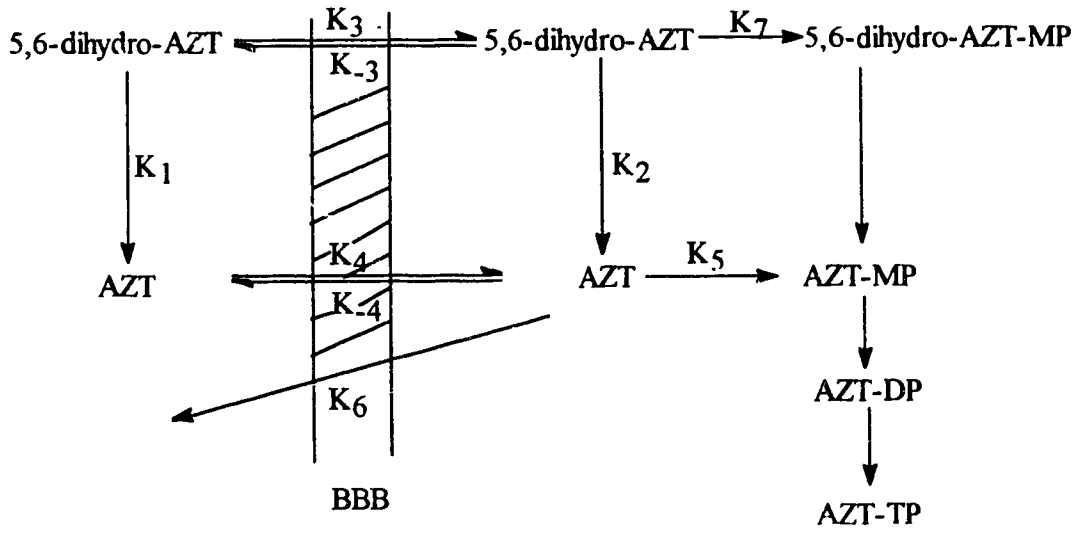


Figure 4.9.4. Diagram of the proposed uptake process for AZT in the brain.

If one assumes that $K_1=K_2$, $K_3=K_{-3}$ and $K_4=K_{-4}$, then the net trapping effect will depend on the relative magnitude of K_5 and K_6, with K_5 leading to accumulation and K_6 leading to decreased concentrations. The net balance will also be influenced by K_7, if this reaction were to occur and by any influence that the prodrug might exert on the organic anion transporter (K_6).

The elevated brain uptake after dosing with the 5,6-dihydro analogs suggests that the net effects lead to accumulation and better therapeutic efficacy is to be expected by utilizing these AZT analogs, but an analysis of levels of ^{14}C-AZT-TP formed from the prodrugs is required for clarification of this situation.

The 5,6-dihydro analogs designed have provided a similar increase in brain uptake of radioactivity to DP-AZT. However, the glycosyl phosphotriester prodrug described earlier provides a higher brain concentration, resulting in a ratio of brain/serum of the prodrug greater than 1 with a significantly improved pharmacokinetic profile of AZT-5'-phosphate in brain compared to that for AZT (peak concentration of 156 nmol/g vs. 5 nmol/g; half-life 24 h vs. 1 h; AUC 4366 nmol·h/g vs. 4 nmol.h/g). Although phosphotriesters provide an increased brain uptake, they are more toxic than AZT in CEM-C113 cells. The clinical potential of these types of compounds remains to be determined.

4.9.3. Comparison of bone radioactivity levels in mice after i.v. injection of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe or [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt.

Bone marrow toxicity is the main side effect resulting from the clinical use of AZT. It is believed to be the result of formation of AMT and a deficiency of dTTP caused by AZT. To reduce the toxicity associated with AZT, the level of AZT in bone marrow has to be minimized. It was important to examine whether the increased brain uptake and decreased clearance observed for 5-halo-6-methoxy-5,6-dihydro analogs of AZT are associated with elevated bone uptake.

Analysis of bone samples showed that the quantities of radioactivity in bone following the administration of AZT, or these 5,6-dihydro analogs of AZT, were about the same (Figure 4.8.5.), suggesting that the 5,6-dihydro analogs do not increase the bone concentration of AZT. Because of the slower clearance and higher brain uptake observed,

if smaller or less frequent doses of the 5,6-dihydro analogs to provide the same therapeutic effect, less toxicity will be expected. In addition, development of resistance is expected to be delayed by smaller doses of AZT since it was reported that resistance tends to develop earlier with AZT dosages of 1200 to 1500 mg/day than dosages of 500 mg/day.

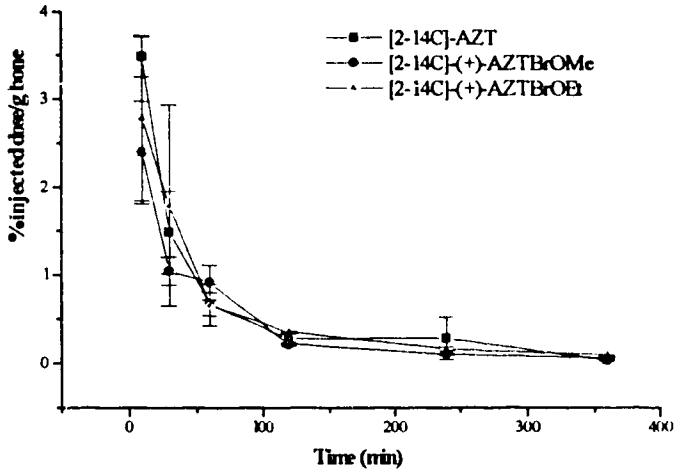


Figure 4.9.5. Bone levels of radioactivity after i.v. injection of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs of AZT (2 μCi, specific activity = 56 mCi/mmol). The data shown are means ± SD, n=3.

4.10. Brain subcellular distribution of radioactivity after administration of [2-^{14}C]- AZT or its [2-^{14}C]- 5-bromo-6-alkoxy-5,6-dihydro analogs.

The biodistribution studies described in Section 4.9. suggest that 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro-3'-azido-3'-deoxythymidines were more effective than AZT for brain targeting, since about three times more radioactivity was trapped in brain tissue 10 min after injection of these prodrugs than for AZT. However, the results obtained from the biodistribution study do not unambiguously prove the presence of AZT, its 5,6-dihydro prodrugs or their metabolites in the cerebral tissue. Penetration of the

nerve terminals is presumably important in the actions of drugs such as AZT. A brain subcellular fractionation study was therefore performed to investigate whether the higher brain uptake of radioactivity resulting from the prodrugs was associated with specific binding of the 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro-3'-azido-3'-deoxythymidines to any specific subcellular components, particularly the P_2 fraction which contains synaptosomes (pinched-off nerve endings). An *ex vivo* study was performed in mice, using literature methods with minor modifications.[311]

4.10.1. Preparation of mouse brain subcellular fractions and analysis of protein.

Four to six mouse brains were combined and subjected to a sequence of centrifugations as illustrated in the Experimental Section (page 75). The P_1, P_2 and P_3 pellets were obtained after 1,000 x *g*, 10,000 x *g* and 100,000 x *g* centrifugations, respectively. The S_3 fraction was the final supernatant. The protein content of each fraction was determined by the Lowry method and the results, defined as the amount of protein in each fraction divided by the total amount of protein, are shown in Figure 4.10.1. The P_1 fraction, which contains nuclei and cell debris contained 13.7% protein. The P_2 fraction, which is a mixture of myelin fragments, synaptosomes and mitochondria, was found to represent 49.3% of the total protein. The P_3 fraction, which is the microsomal fraction, contained 12.0% protein and the S_3 fraction, which is the soluble fraction, contained 25.1% protein. These results indicated that about half of the total brain protein was recovered from the P_2 fraction. The protein contents in subcellular fractions P_1, P_2, P_3, and S_3 from guinea pig brain were reported to be 30, 33, 14 and 23%, respectively.[311] The differences in P_1 and P_2 between our results and the reported results are possibly due to the species difference or the degree of homogenization. The more the brain tissue is homogenated, the less is expected in this fraction, since the P_1 contains nuclei and cell debris.

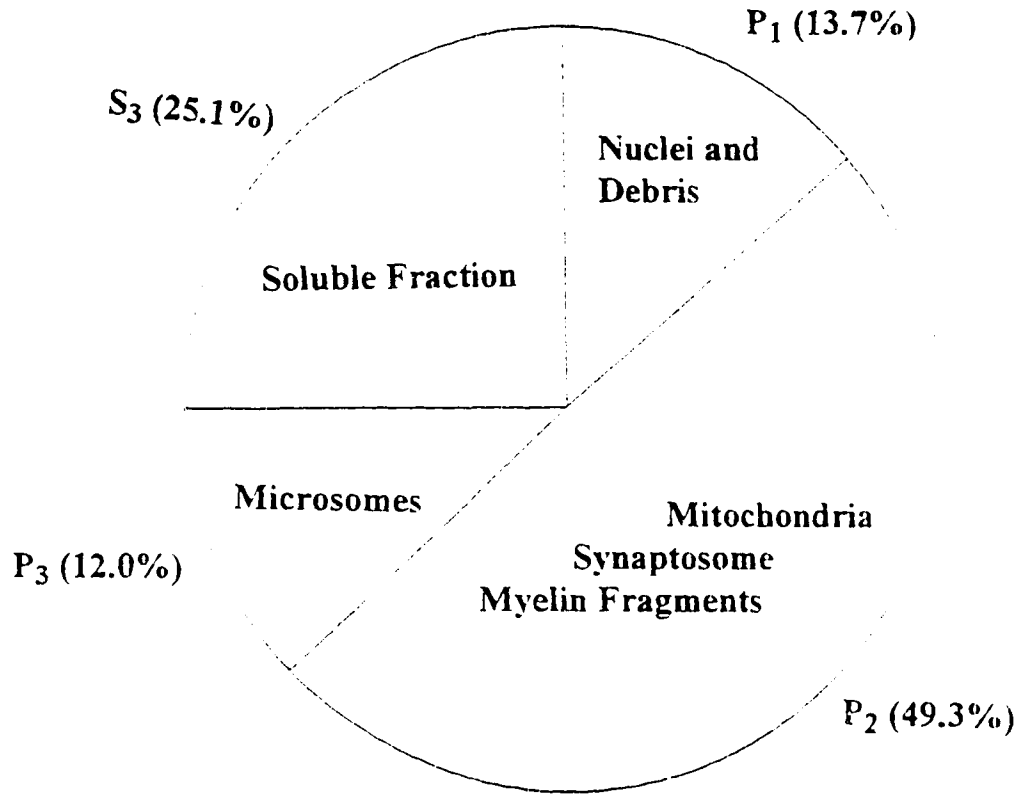


Figure 4.10.1. Protein distribution in mouse brain subcellular fractions.

4.10.2. Subcellular distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-alkoxy-5,6-dihydro analogs in brain after jugular vein injection.

The subcellular distributions of [2-^{14}C]-AZT, [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe, and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt in brain were investigated in Balb/c mice after injecting the compound via the jugular vein. Distribution studies after tail vein injection of [2-^{14}C]-AZT (2 μCi, specific activity 56 mCi/mmole), [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe (2 μCi, specific activity 56 mCi/mmole), and [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt (2 μCi, specific activity 56 mCi/mmole) have indicated that less than 5% of the injected dose was present in blood after 10 min (see Tables 4.9.1.-4.9.4.).

Jugular vein injections were expected to increase the amount delivered to the brain. For this study, the brains from 5 mice were pooled prior to fractionation to increase the accuracy of the analysis. The mouse brains were collected 10 min after the jugular vein injection and fractionated into P_1, P_2, P_3 and S_3 fractions by the centrifugation method. The quantity of radioactivity and protein content were determined for each fraction. The distribution of radioactivity as well as RSC (relative specific concentration) for the compounds investigated are presented in Figure 4.10.2. and Table 4.10.1. A value of RSC greater than 1 represents a considerable localization.

The results obtained indicate that the subcellular distribution of radioactivity in the brain after jugular vein injection of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs was very similar. There was a considerable amount of radioactivity in the S_3 fraction, suggesting that there was no specific binding of the test compounds to any subcellular component in P_1, P_2, or P_3 or that the binding was not very "tight". Although a similar brain subcellular distribution pattern in brain was observed after [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs (see column B in Table 4.10.1.), higher radioactivity levels were recovered from brain after [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT than after [2-^{14}C]-AZT, which suggested that the 5-bromo-6-alkoxy-5,6-dihydro analogs of AZT enter brain at a faster rate than AZT.

Table 4.10.1. Distribution of radioactivity after administration of [^{14}C]-AZT and [^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in subcellular fractions of mouse brain (pooled samples from 5 mice).

	Protein distribution (A)[a]			**Radioactivity distribution (B)[b]**			**CPM/mg protein (C)[c]**			**Relative Specific Concentration (RSC=B/A)**		
	AZT	**AZTBrOMe**	**AZTBrOEt**	**AZT**	**AZTBrOMe**	**AZTBrOEt**	**AZT**	**AZTBrOMe**	**AZTBrOEt**	**AZT**	**AZTBrOMe**	**AZTBrOEt**
P_1	15.9%	13.3%	11.9%	14.6%	4.6%	5.2%	110	208	390	0.92	0.34	0.44
P_2	51.7%	53.1%	43.0%	7.6%	5.6%	7.7%	28	64	159	0.15	0.11	0.18
P_3	9.6%	8.7%	17.6%	3.4%	1.4%	2.4%	47	97	119	0.35	0.16	0.14
S_3	22.8%	24.9%	27.5%	74.5%	88.4%	84.7%	1000	2145	2735	3.30	3.55	3.10

[a]% total protein recovered.

[b]% total radioactivity recovered.

[c]radioactivity (CPM)/mg protein.

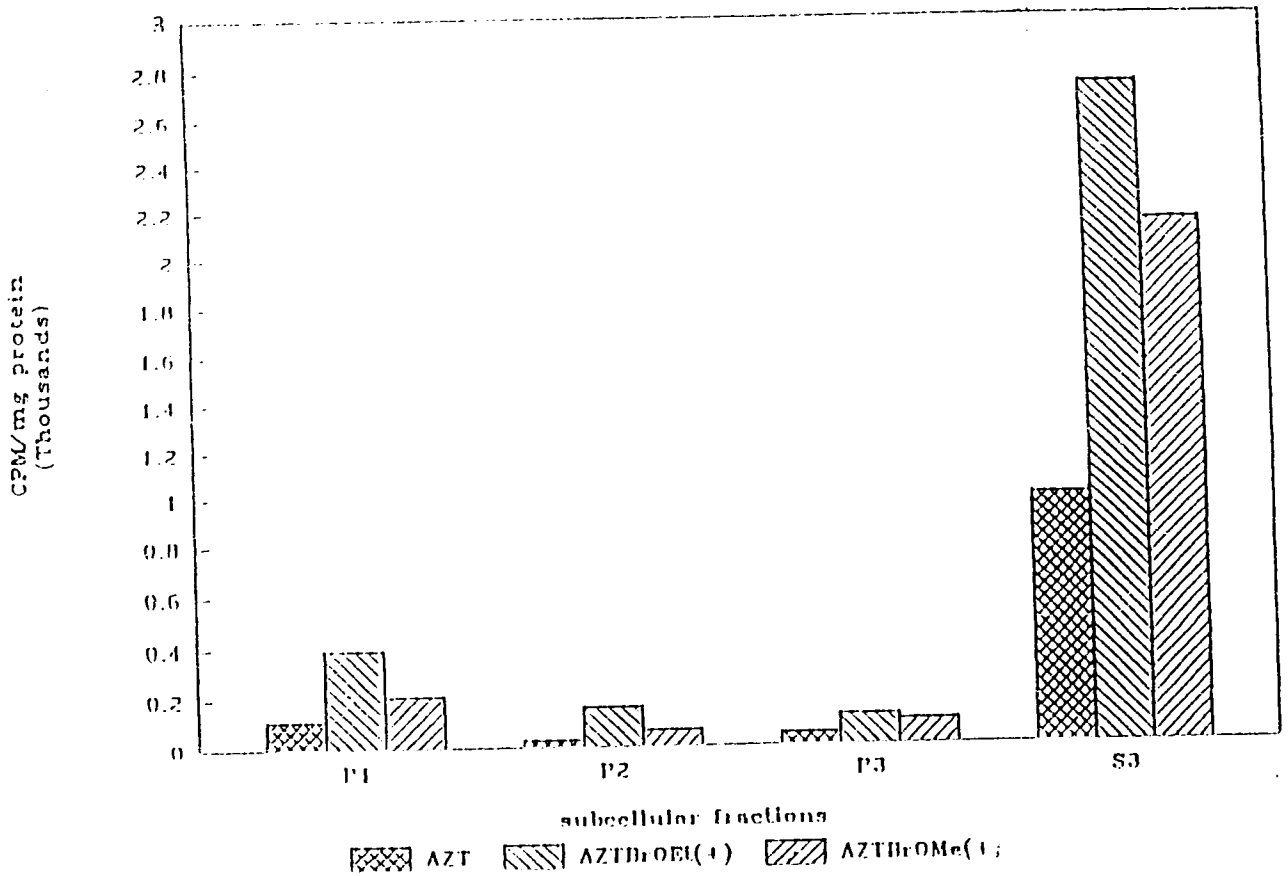


Figure 4.10.2. Distribution of [2-^{14}C]-AZT and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in subcellular fractions of mouse brain (2 μCi, specific activity 56 mCi/mmole; pooled brain samples from 5 mice).

4.11. Regional distribution of [2-^{14}C]-AZT and its [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs in brain.

Although the precise cause of the HIV-related CNS disorder is unknown, histological studies indicate that loosely organized cellular infiltrates including macrophages, microglia, astrocytes, and scattered multinucleated cells are present in the AIDS dementia complex.[269,270] These cellular infiltrates are commonly found in basal ganglia, brain stem, and subcortical white matter, with the extent of infiltration correlating with the degree of dementia Although the mode of HIV entry into the CNS remains unclear, HIV infection of the brain is suggested by the presence of HIV-1 antigen within the cerebrospinal fluid (CSF) and by intra-BBB synthesis of HIV-1-specific antibodies.[270] As reported in the literature[92], AZT distributes into CSF with CSF/plasma concentration ratios of 0.24-0.85. However, rat studies showed that less than 1.0% of injected AZT[134,135] penetrated into brain. It has been observed that [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT resulted in higher concentrations of radioactivity in brain than for [2-^{14}C]-AZT, but that no differences in subcellular localization of radioactivity occurred within the brain. We then conducted a study of the regional distribution of [2-^{14}C]-AZT and [2-^{14}C]-trans-(+)-(5R,6R)-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in mouse brain. The mouse brains collected 10 min after jugular vein injection were dissected into cerebellum, pons and medulla, hypothalamus, hippocampus, striatum, cortex and the rest of brain. Since the weight of each individual part was small and associated radioactivity was low, the individual parts from four (for AZT) and two (for 5,6-dihydro analogs) mice were pooled. They were weighed wet and analyzed by the standard combustion/liquid scintillation counting method described in Section 3.2.8.3 (page 73). The results calculated as CPM/mg regional tissue are shown in Table 4.11.1. The results obtained indicate that radioactivity resulting from all three compounds is relatively uniformly distributed into all regions of brain examined

with the exception of the hypothalamus where a higher concentration (about 2-fold compared to other regions) was observed. of [2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt, levels of radioactivity in the hippocampus were also high, similar to those in the hypothalamus. The mechanism by which the HIV virus enters the brain, and whether the virus is localized preferentially in one part of the brain are not well understood. The results obtained in this study may serve as the basis for future investigation.

Table 4.11.1. Regional study of [2-^{14}C]-AZT and [2-^{14}C]-5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs of AZT in mouse brain (2 μCi, specific activity 56 mCi/mmole).

	Radioactivity (CPM/mg) from pooled samples		
	[2-^{14}C]-AZT[a]	**[2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOMe[b]**	**[2-^{14}C]-trans-(+)-(5R,6R)-AZTBrOEt[b]**
cerebellum	13.4	71.3	64.9
pons & medulla	14.8	65.4	60.6
hypothalamus	20.5	130.3	108.0
hippocampus	14.2	70.1	107.9
striatum	14.2	86.3	83.4
cortex	12.1	69.8	65.2
rest of brain	12.9	69.6	81.6

[a] Sample was pooled from four individual mice.
[b] Sample was pooled from two individual mice.

5. Summary and Conclusions

The development of anti-HIV therapy has resulted in the introduction of four nucleoside drugs for clinical use, i.e. AZT, ddI, ddC and d4T. These drugs all function to inhibit HIV RT, which plays a key role in replication of the virus. Recognition of toxicity, fast blood clearance and limited brain uptake, as well as the appearance of resistance, suggests that more potent agents with less toxicity, increased brain delivery, and improved pharmacokinetic profiles are needed. Current research underway includes the development of new agents with different mechanisms of action as well as improvement of the drugs already shown to be active. With regard to improving the therapeutic efficacy of current drugs, research to develop prodrugs of AZT and other anti-HIV nucleoside analogs has been very active. 5-Halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T have been synthesized as new anti-HIV agents and novel prodrugs for AZT, FLT and d4T.[313-315] Anti-HIV test results suggested that their activity against the HIV-1 virus *in vitro* is dependent on the nature of substituents at both C^5 and C^6 positions.[313-315] We have studied these 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T as potential lipophilic prodrugs. From the investigations described in this thesis, the following summary and conclusions can be made:

1. The 5,6-dihydro analogs are equal or less potent anti-HIV agents than their corresponding parent compounds *in vitro,* depending upon the substituents at C^5 and C^6 of the 5,6-dihydro analogs. The 5-iodo (or bromo) analogs are generally more potent than their corresponding 5-chloro analogs. The 6-methoxy analogs are generally more active than the analogs with other alkoxy groups at the C^6 position. The stability study of 5-halo-6-methoxy-5,6-dihydro analogs of AZT in cell culture medium (MEMS/10%FBS) showed that the 5-iodo (or bromo)-6-methoxy-5,6-dihydro analogs of AZT gradually convert to AZT in the cell culture medium, whereas the 5-chloro-6-methoxy-5,6-dihydro

analog of AZT does not convert to AZT under these conditions. This indicates that the EC_{50} and IC_{50} values obtained for the 5-bromo (or iodo) analogs reflect the conversion from the 5,6-dihydro analogs to their corresponding parent compounds during *in vitro* incubation and that the EC_{50} and IC_{50} of the 5-chloro analogs are not due to a prodrug effect.

2. The 5,6-dihydro analogs of AZT, FLT and d4T are more lipophilic than their corresponding parent drugs. The determination of partition coefficients in the 1-octanol/water system indicated that 5-halo-6-methoxy-5,6-dihydro analogs of AZT are more lipophilic (P = 3.3-18.81) than AZT (P = 1.29). The 5-halo-6-methoxy-5,6-dihydro analogs of FLT and d4T are three to ten-fold and eight to twenty-eight fold more lipophilic than FLT (P = 0.5) and d4T (P = 0.12), respectively. Individual diastereomers possess different lipophilicities, suggesting that the configurations at C^5 and C^6 are determinants. The retention times of 5,6-dihydro analogs of AZT are significantly longer than that of AZT on reverse phase HPLC (10-24 min vs. 7.5 min) using methanol:water (6:4) at 1 mL/min, again indicating that the 5,6-dihydro analogs are more lipophilic than AZT. There is a linear correlation (log P = 0.061 t_R - 0.2215 [R^2 = 0.90]) between the partition coefficients (log P) and the retention times (t_R) for AZT and its 5-halo-6-methoxy-5,6-dihydro analogs. This may provide a tool for predicting partition coefficients of other compounds in this series (5-halo-6-alkoxy-5,6-dihydro analogs of AZT) from their retention times in reverse phase HPLC under the same conditions.

3. The 5,6-dihydro analogs, in most cases, have increased affinity than AZT for the mouse erythrocyte NBMPR-sensitive nucleoside transporter. Nucleosides are mainly transported across cell membranes by facilitated mechanisms. The NBMPR-sensitive nucleoside transporter (NT) is one of the better characterized transport systems, and accepts a wide range of substrates. Thymidine is known to traverse human erythrocyte membranes by this system. However, AZT and d4T as well as other ddNs have been shown to translocate through cell membranes by passive diffusion. FLT has been shown

to cross cell membrane by two mechanisms (NBMPR-sensitive and -insensitive). It is also known that thymidine transport inhibitors potentiate the efficacy of AZT.

The interaction of AZT and its 5,6-dihydro analogs with the NBMPR-sensitive NT has been investigated by determination of the inhibition constants (K_i) of AZT and its 5,6-dihydro analogs from thymidine influx competition experiments in a simple model (mouse erythrocytes). The K_i value represents the relative affinity of test compounds for the NBMPR-sensitive transport system and the results indicate that trans-5-halo-6-methoxy-5,6-dihydro analogs have higher affinity (K_i = 0.2-0.4 mM) for the NBMPR-sensitive NT system than does AZT (K_i = 1.3 mM). In contrast, cis-(+)-(5S,6R)-AZTClOMe (K_i >> 1.5 mM) has a decreased affinity relative to AZT. It appears that the affinity of these 5,6-dihydro analogs for the NBMPR-sensitive NT system is dependent on the configuration of substituents at the C^5 and C^6 positions. With increased affinity for the NBMPR-sensitive nucleoside transport system, these analogs may interfere with the cellular uptake of thymidine. However it is not known if this will result in significantly increased efficacy of AZT in a clinical setting.

4. The 5,6-dihydro analogs are not subject to phosphorolysis by *E. coli* thymidine phosphorylase, suggesting that the 5,6-dihydro analogs would be stable to phosphorolysis *in vivo*. Phosphorolysis of nucleoside analogs (with production of nucleobase and 1-phosphate ribose or 1-phosphate-2'-deoxy-ribose) is a catabolic reaction. Although AZT is stable toward thymidine phosphorolysis, d4T, DU and EDU underwent 10, 57 and 12% phosphorolysis. All 5-halo-6-methoxy (or azido)-5,6-dihydro analogs of AZT, d4T, DU and EDU examined were found to be stable toward phosphorolysis by *E. coli* thymidine phosphorylase.

5. *In vitro* conversion of the 5,6-dihydro analogs to AZT, FLT and d4T is dependent upon the nature of the halogen at the C^5 position. The conversion from 5,6-dihydro uracils to the parent uracils maybe a thiol-mediated process. Regeneration of AZT, FLT and d4T from their 5-halo-6-methoxy-5,6-dihydro analogs was investigated

following incubation of the individual 5,6-dihydro analog with glutathione, mouse whole blood or a mouse soluble liver enzyme fraction at 37°C. The results showed that both 5-iodo and 5-bromo-6-methoxy-5,6-dihydro analogs convert to their corresponding parent compounds to different degrees in the order of I > Br, whereas 5-chloro-6-methoxy-5,6-dihydro analogs were stable. All of the 5-halo-6-methoxy-5,6-dihydro analogs of AZT examined are stable upon incubation with phosphate buffer at 37°C for at least 24 h. A dependency of the % regeneration of the 5,6-double bond upon the nature of the halogen at the C^5 position has been demonstrated.

6. The 5-bromo (or iodo)-6-methoxy-5,6-dihydro analogs provided a considerable amount of AZT in mouse blood after i.v. injection. The prodrugs convert to AZT *in vivo*, and this conversion is dependent upon the nature of halogen at C^5 ($I \geq Br > Cl$). 5-Bromo (or iodo)-6-methoxy-5,6-dihydro analogs (144 μmol/kg) gave rise to AZT blood concentrations similar to those measured after equi-molar doses of AZT. On the other hand, the regeneration of AZT from its 5-chloro-6-methoxy-5,6-dihydro analog (240 μmol/kg) is about 10 % of that for the bromo or iodo analogs at 15 min after administration of 144 μmol/kg, and AZT was not detectable after 90 min.

7. The 5,6-dihydro analogs enter the brain more readily than AZT. Increased lipophilicity should enable the 5,6-dihydro analogs to enter the brain more effectively. The biodistributions of ^{14}C labeled AZT and its 5-bromo-6-alkoxy (methoxy and ethoxy)-5,6-dihydro analogs were examined in mice after i.v. injection. Radioactivity recovered from brain for the [2-^{14}C]-5-bromo-6-methoxy or 6-ethoxy-5,6-dihydro analogs was approximately three times that after [2-^{14}C]-AZT was administered. The AUC for the [2-^{14}C]-5,6-dihydro analogs (53.8 and 51.5 % ID·min/g for AZTBrOMe and AZTBrOEt) in brain were significantly higher than that for [2-^{14}C]-AZT (17.4 % ID·min/g).

The radioactivity present in blood after dosing with the 5,6-dihydro analogs is significantly higher than that for AZT for a longer time period (after 2 h), suggesting that the 5,6-dihydro analogs undergo slower clearance or that this reflects a depot effect. In

addition to the elevated blood and brain levels of radioactivity, the radioactivity recovered from bone was similar after administration of equimolar doses of AZT and the 5,6-dihydro analogs, which may indicate an improved therapeutic index for the prodrug.

8. Examination of subcellular fractions of mouse brain after jugular vein injection of ^{14}C labeled AZT or its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs, suggested that both AZT and the 5,6-dihydro analogs investigated had little specific binding to the particulate subcellular fractions P_1 (nuclei, cell debris), P_2 (mitochondria, synaptosome) and P_3 (microsomes). There was a considerable localization in the soluble fraction (S_3). Increased radioactivity was present in all the fractions after the prodrug administration relative to that after AZT administration.

9. Measurement of radioactivity in various regions of mouse brain after jugular vein injection of ^{14}C labeled AZT or its 5-bromo-6-methoxy (or ethoxy)-5,6-dihydro analogs indicated that radioactivity from both AZT and the prodrugs exhibited very little preferential regional localization in brain.

Overall, based on the the *in vitro* and *in vivo* evaluations of selected 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT, FLT and d4T, it is clear that the 5,6-dihydro analogs represent a valuable approach to the design and development of prodrugs for nucleoside RT inhibitors.

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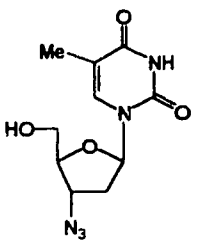
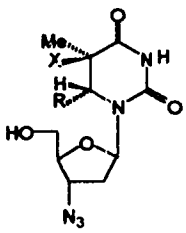
Appendix

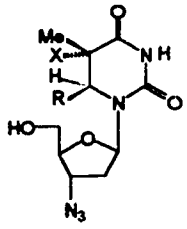
Table 1. *In vitro* anti-HIV activity and selectivity of 5-halo-6-alkoxy (or azido)-5,6-dihydro analogs of AZT in HIV-1 infected CEM cells.



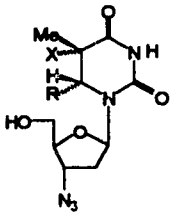
AZT



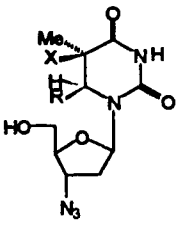
1,3,5,7,9,12-18,20
trans-(+)-(5R,6R)



2,6,8,10,19,21
trans-(-)-(5S,6S)



4,11,22
cis-(+)-(5S,6R)



23
cis-(-)-(5R,6S)

No.	X	R	Configuration	IC_{50} (M)[a]	EC_{50} (M)[b]	T. I[d]
1	Br	OCH_3	5R,6R	1.72×10^{-5}	3.27×10^{-9}	5260
2	Br	OCH_3	5S,6S	4.25×10^{-5}	2.80×10^{-7}	152
3,4[c]	Cl	OCH_3	5R,6R; 5S,6R	$>8.98 \times 10^{-4}$	5.79×10^{-6}	155
5	I	OCH_3	5R,6R	1.87×10^{-5}	3.17×10^{-9}	5899
6	I	OCH_3	5S,6S	6.42×10^{-6}	5.15×10^{-9}	1246
7	Br	OC_2H_5	5R,6R	1.85×10^{-5}	6.75×10^{-9}	2741
8	Br	OC_2H_5	5S,6S	2.22×10^{-5}	2.37×10^{-8}	937
9	Cl	OC_2H_5	5R,6R	$> 2.00 \times 10^{-4}$	Inactive	ND
10	Cl	OC_2H_5	5S,6S	$> 2.00 \times 10^{-4}$	Inactive	ND
11	Cl	OC_2H_5	5S,6R	$> 2.00 \times 10^{-4}$	Inactive	ND
12	Br	$OCH(CH_3)_2$	5R,6R	$> 2.00 \times 10^{-4}$	5.72×10^{-6}	35
13	Cl	$OCH(CH_3)_2$	5R,6R	$> 2.00 \times 10^{-4}$	Inactive	ND
14	Br	$O(CH_2)_7CH_3$	5R,6R	1.24×10^{-5}	8.56×10^{-7}	14
15	Cl	$O(CH_2)_7CH_3$	5R,6R	3.75×10^{-5}	Inactive	ND
16	Br	$O(CH_2)_{15}CH_3$	5R,6R	1.10×10^{-5}	Inactive	ND
17	Cl	$O(CH_2)_{15}CH_3$	5R,6R	3.10×10^{-5}	Inactive	ND
18,19[c]	Br	N_3	5R,6R;5S,6S	1.76×10^{-5}	2.0×10^{-4}	< 1
20-23[c]	Cl	N_3	5R,6R; 5S,6S; 5S,6R;5R,6S	3.5×10^{-4}	1.49×10^{-6}	235
AZT				5×10^{-4}	3×10^{-9}	>> 10,000

continued

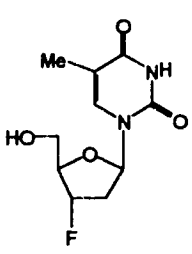
[a] The IC_{50} value is the test drug concentration which results in a 50% survival of uninfected untreated control CEM cells (e.g. cytotoxicity of the test drug).

[b] The EC_{50} value is the test drug concentration which produces a 50% survival of HIV-1 infected cells relative to uninfected untreated controls (e.g. in vitro anti-HIV activity).

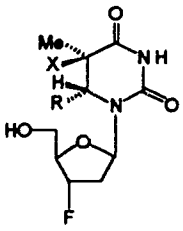
[c] Tested as a mixture of diastereomers.

[d] Therapeutic index = IC_{50}/EC_{50}.

Table 2. *In vitro* anti-HIV activity and selectivity of 5-halo-6-methoxy (or azido)-5,6-dihydro analogs of FLT in HIV-1 infected CEM cells.

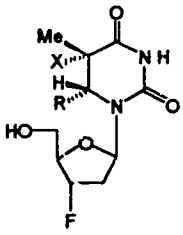


FLT



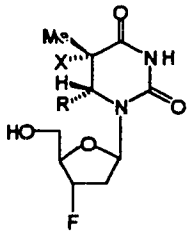
1,3,5,7

trans-(+)-(5R,6R)



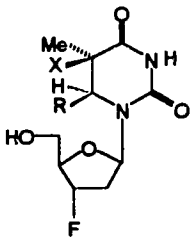
2,6,8

trans-(-)-(5S,6S)



4

cis-(+)-(5S,6R)



9

cis-(-)-(5R,6S)

No.	X		Configuration	IC_{50} (M)[a]	EC_{50} (M)[b]	T. I.[d]
1	Br	OCH_3	5R,6R	1.72×10^{-6}	5.25×10^{-9}	327
2	Br	OCH_3	5S,6S	9.72×10^{-6}	3.25×10^{-9}	2990
3	Cl	OCH_3	5R,6R	$> 8.0 \times 10^{-4}$	5.55×10^{-6}	144
4	Cl	OCH_3	5S,6R	$> 8.0 \times 10^{-4}$	3.79×10^{-6}	211
5	I	OCH_3	5R,6R	5.73×10^{-5}	5.00×10^{-5}	1.15
6	I	OCH_3	5S,6S	1.22×10^{-5}	3.75×10^{-9}	3253
7,8,9[c]	Br	N_3	5R,6R;5S,6S; 5R,6S	1×10^{-4}	1.45×10^{-8}	7143
FLT					1×10^{-9}	ND

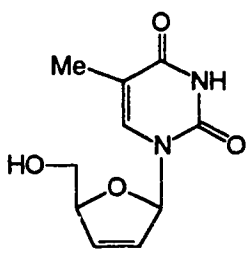
[a] The IC_{50} value is the test drug concentration which results in a 50% survival of uninfected untreated control CEM cells (e.g. cytotoxicity of the test drug).

[b] The EC_{50} value is the test drug concentration which produces a 50% survival of HIV-1 infected cells relative to uninfected untreated controls (e.g. in vitro anti-HIV activity).

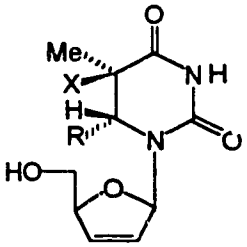
[c] Tested as a mixture of diastereomers.

[d] Therapeutic index = IC_{50}/EC_{50}.

Table 3. In vitro anti-HIV activity and selectivity of 5-halo-6-methoxy-5,6-dihydro analogs of d4T in HIV-1 infected CEM cells.

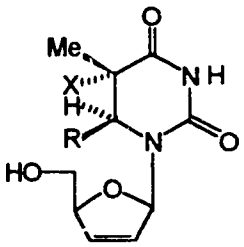


d4T



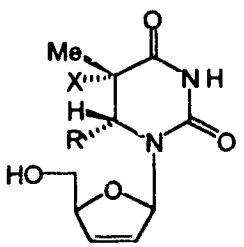
1,3,5

trans-(+)-(5R,6R)



2,4

trans-(-)-(5S,6S)



6

cis-(+)-(5S,6R)

No.	X	R	Configuration	IC_{50} (M)[a]	EC_{50} (M)[b]	T. I.[d]
1,2[c]	Br	OCH_3	5R,6R; 5S,6S	> 1.28×10^{-4}	5.46×10^{-5}	2.34
3,4[c]	Cl	OCH_3	5R,6R; 5S,6R	> 1.03×10^{-3}	3.75×10^{-4}	2.75
5,6[c]	I	OCH_3	5R,6R; 5S,6S	6.6×10^{-5}	3.76×10^{-7}	176
d4T				3×10^{-4}	1.20×10^{-7}	25

[a] The IC_{50} value is the test drug concentration which results in a 50% survival of uninfected untreated control CEM cells (e.g. cytotoxicity of the test drug).

[b] The EC_{50} value is the test drug concentration which produces a 50% survival of HIV-1 infected cells relative to uninfected untreated controls (e.g. in vitro anti-HIV activity).

[c] Tested as a mixture of diastereomers.

[d] Therapeutic index = IC_{50}/EC_{50}.