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Targeted drug delivery to the brain via phosphonate derivatives II. Anionic chemical delivery system for zidovudine (AZT)

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Abstract

The previously described anionic chemical delivery system (aCDS) approach (Somogyi, G., Nishitani, S., Nomi, D., Buchwald, P., Prokai, L., Bodor, N., 1997. Targeted drug delivery to the brain via phosphonate derivatives. I. Design, synthesis and evaluation of an anionic chemical delivery system for testosterone. Int. J. Pharm. (166, 15–26) was applied for brain-targeted delivery of AZT. For this system, the whole designed metabolic sequence, ending with release of the active drug at the targeted organ, was completed. As a less hindered ester function was built into this aCDS, cleavage by esterases, the first metabolic step in the decomposition process, was fairly rapid. The negatively charged decomposition product (AZT-P^-) could be detected for about 48 h in different organs. In vitro experiments proved that AZT is released from this phosphonate derivative after phosphorolytic attack, the second metabolic step of the designed sequence, but only at a slow rate. While the phosphonate derivative of a secondary hydroxyl group proved completely resistant to such phosphorolytic attacks, alkaline phosphatase, but not phosphodiesterase, was able to cleave the P–O bond of the phosphonate derivative at the primary hydroxyl group in this system. After i.v. administration of AZT-aCDS in rabbits, AZT could be detected in the brain, albeit only at very low concentrations. Even if sufficiently high drug levels could not be delivered with the present aCDSs, the sequence was completed, the rate of metabolism was controllable, and the approach is flexible enough; therefore, further, adequate manipulations might render such anionic chemical delivery systems into a useful addition of the drug targeting arsenal. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Brain-targeted delivery; Anionic chemical delivery system (aCDS); Azidothymidine (AZT); Alkyl phosphatase and phosphodiesterase

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1. Introduction

Within the general principles of retrometabolic drug design (Bodor, 1994; Bodor and Buchwald, 1997), a novel anionic chemical delivery system (aCDS) approach, involving an (acyloxy)alkyl phosphonate targetor moiety (Bodor, 1995), has been developed and applied recently for brain-targeted delivery of testosterone (Somogyi et al., 1997). Such enzymatic physical-chemical based CDSs rely on a designed metabolic sequence that first transforms the systemically administered, lipophilic CDS into a hydrophilic intermediate that will be locked inside the brain by the blood–brain barrier (BBB) and then provides a sustained release of the active drug at its site of action (Bodor, 1987, 1994; Bodor and Buchwald, 1997). For the testosterone-aCDS that uses a (pivaloyloxy)methyl (POM) phosphonate ester targetor moiety, we have found that the first ester is cleaved through a hydrolytic reaction yielding the negatively charged, 'locked in' intermediate, but dephosphorylation of the phosphonate derivative at the secondary hydroxyl group, the enzymatic step that should release the active drug, does not occur in a detectable manner.

Here we report work on a similar aCDS for AZT (3′-azido-3′-deoxythymidine, zidovudine), where attachment of the (acyloxy)alkyl phosphonate targetor could be carried out at a primary hydroxyl group. There are several other reasons to investigate a brain-targeted delivery system for AZT. Since in the brain of patients with AIDS dementia, HIV-1 virus, proviral DNA, viral nucleic acid, viral antigens, and HIV-1 virions are present (McArthur, 1987), an ideal chemotherapeutic agent for HIV-1 should penetrate the central nervous system (Yarchoan and Broder, 1987), even if it is not certain whether or not the pathology of AIDS dementia complex is caused by a direct infection of the brain or by an indirect mechanism. Despite entry into the human cerebrospinal fluid (CSF) (Klecker et al., 1987), AZT, like most polar nucleosides, does not readily cross the blood–brain barrier, e.g. radioactive AZT was not shown to penetrate in significant amounts into the brain of rats (Ellison et al., 1988). This modified riboside has been shown to be useful in improving the neuropsychiatric course of AIDS encephalopathy in a few patients, but the doses required to elicit this improvement precipitate severe anemia, bone marrow suppression, and other toxic effects (Yarchoan et al., 1987; Mitsuya et al., 1990). In an effort to ameliorate the prognosis of AIDS encephalopathy, the dihydropyridine–pyridinium salt redox-type CDS approach (Bodor, 1987) was already applied to AZT with promising results (Brewster et al., 1988; Torrence et al., 1988; Gallo et al., 1989; Gogu et al., 1989; Little et al., 1990). Since the phosphonate derivative of a secondary hydroxyl group proved resistant to phosphorolytic attacks in the anionic chemical delivery system for testosterone, we investigated the possibilities of this delivery system for AZT, where attachment of the (acyloxy)alkyl phosphonate targetor was carried out at a primary alcohol, and cleavage of this less hindered P–O bond is more likely.

2. Materials and methods

2.1. Chemistry

Melting points (MP) were obtained using a Fisher–Johns melting points apparatus and are uncorrected. Mass spectra (MS) were recorded by a Kratos Analytical MS80RFA instrument using fast atom bombardment (FAB). Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded in a Varian EM390 (90 MHz) spectrometer. Samples were dissolved in an appropriate deuterated solvent and chemical shifts (δ) reported in ppm relative to an internal standard (tetramethylsilane, TMS). Elemental analyses were performed by Atlantic Microlabs (Atlanta, GA). All starting materials were of reagent grade and obtained from Aldrich Chemical Co. (Milwaukee, WI). 3′-Azido-3′-deoxythymidine (AZT) was obtained from Pharmatec Inc. (Alachua, FL). Merck Kieselgel 60 (70–230 Mesh ASTM) and Aldrich Florisil (100–200 Mesh) were used for column chromatography. Phosphodiesterase I (E.C. 3.1.4.1) type IV (0.028 units/mg solid), and alkaline phosphatase (E.C. 3.1.3.1, 2000 units) were used for enzymatic assay, and were purchased from Sigma Chem. Co. (St. Louis, MO).

2.2. Synthesis

2.2.1. Chloromethyl hexanoate (1)

To a mixture of hexanoyl chloride (25 g, 0.186 mol) and paraformaldehyde (5.58 g, 0.186 mol), a catalytic amount (550 mg) of zinc chloride was added in an ice bath. After the resulting exothermic reaction subsided, the mixture was heated at 90–100°C for 4.5 h. Purification by distillation at reduced pressure gave 22.79 g of a colorless liquid in 75% yield; boiling point 37–40°C/0.55 mmHg. NMR ($CDCl_3$): 0.90 (t, 3H, $J = 6$ Hz), 1.20–1.80 (m, 6H), 2.37 (t, 2H, $J = 7$ Hz), 5.67 (s, 2H).

2.2.2. Iodomethyl hexanoate (2)

Chloromethyl hexanoate (1) (205 mg, 1.25 mmol) was stirred with sodium iodide (900 mg, 6.0 mmol) in 3 ml of dry acetone for 4 h at room temperature. The insoluble (sodium chloride) material was removed by filtration and washed with fresh acetone. The filtrate was evaporated; hexane and 5% aqueous sodium thiosulfate solution were added to the residue. After the mixture was thoroughly shaken, the organic layer was separated and washed with 5% aqueous sodium thiosulfate solution, then dried over sodium sulfate. The solvent was evaporated. Iodomethyl hexanoate, $CH_3(CH_2)_4COOCH_2I$, was obtained as a yellow oil in 78% yield (250 mg). NMR ($CDCl_3$): 0.90 (t, 3H, $J = 6$ Hz), 1.20–1.80 (m, 6H), 2.30 (t, 2H, $J = 7$ Hz), 5.89 (s, 2H). The compound was used for the next reaction without further purification.

2.2.3. 5′-(3′-Azido-3′-deoxythymidyl) methylphosphonate (AZT-P^-, 3)

AZT (zidovudine, 3′-azido-3′-deoxythymidine) (10.0 g, 37.4 mmol), sodium carbonate (11.9 g, 112 mmol), methylphosphonic dichloride (14.9 g, 112 mmol), and dry acetone (50 ml) were combined in a round-bottom flask under a stream of nitrogen; the mixture was stirred at room temperature for 17 h and then cooled. The acetone solvent was dried with anhydrous K_2CO_3, and then distilled (b.p. 56.2°C) before using. To the ice-cooled, stirred residual mixture, water (4.0 ml, 225 mmol) was added dropwise, followed by 60 ml of methanol. To the resultant suspension, Florisil® (100 g) was added, and the mixture was evaporated to dryness. The crude material was purified by column chromatography on Florisil® (200 g) with dichloromethane–methanol (20:1 to 1:1 gradient) as eluent to give 4.33 g of a crude amorphous solid. The crude solid was dissolved in methanol (21.6 ml), then 216 ml of ether were added. The formed precipitate was collected by filtration and washed with ether. The resulting pale yellow amorphous solid was dried in vacuum and used for the next reaction without further purification. Yield: 4.53 g (42%). NMR (MeOH-d_4): 1.32 (d, 3H, $J = 17$ Hz), 1.91 (s, 3H), 2.25–2.50 (m, 2H), 3.85–4.15 (m, 3H), 4.25–4.50 (m, 1H), 6.17 (t, 1H, $J = 6$ Hz), 7.64 (s, 1H). NMR (DMSO-d_6): 1.5 (d, 3H, $J = 17$ Hz), 1.80 (s, 3H), 2.20–2.45 (m, 2H), 3.75–4.05 (m, 3H), 4.25–4.60 (m, 1H), 6.10 (t, 1H, $J = 7$ Hz), 7.72 (s, 1H).

2.2.4. 5′-(3′-Azido-3′-deoxythymidyl) hexanoyloxymethyl methylphosphonate (AZT-aCDS, 4)

AZT-P^- (3) (4.5 g, 13 mmol), cesium fluoride (4.41 g, 29 mmol), freshly prepared iodomethyl hexanoate (6.68 g, 26 mmol), and dimethylformamide (45 ml) were mixed under a stream of nitrogen and stirred at room temperature for 20 h. The reaction mixture was then poured into 300 ml of ether and washed successively with water (100 ml), 5% aqueous sodium thiosulfate solution (100 ml), and again with water (100 ml). Each aqueous layer was extracted with one 100-ml portion of ether. The organic layers were combined, dried over magnesium sulfate, filtered, and concentrated to give 3.95 g of brown oil. The crude material was purified by column chromatography on silica gel (40 g) using hexane–ethyl acetate (1:1 to 0:1 gradient) as eluent to give 1.03 g of a slightly yellow viscous oil in 16.7% yield. MS: m/z 474 (MH^+). NMR ($CDCl_3$): 0.89 (t, 3H, $J = 6$ Hz), 1.10–1.85 (m, 6H), 1.62 (d, 3H, $J = 18$ Hz), 1.92 (s, 3H), 2.20–2.50 (m, 4H), 5.66 (d, 2H, $J = 13$ Hz), 6.14 (t, 1H, $J = 6$ Hz), 7.26 (s, 1H), 9.40 (bs, 1H). Elemental analysis for $C_{18}H_{28}N_5O_8P \times 1.5H_2O$. Theory: C, 43.20; H, 6.24; N, 13.99. Found: C, 42.94; H, 5.95; N, 14.12.

2.3. Analytical method

The HPLC (UV/VIS detection) method of Little et al. (1990) was adapted and modified for the present work. The chromatographic analysis was performed in a system consisting of Spectra-Physics (Palo Alto, CA) SP 8810 solvent delivery system, SP 8780 auto sampler, SP 8456 UV/VIS variable wavelength detector operated at 266 nm, and SP 4290 integrator. The column was a 3μ Supelco LC-8-DB (7.5 cm, 4.6 mm i.d.) with a 15-mm long (3μ) LC-8-DB guard column. The mobile phase consisted of 10% acetonitrile, 90% 0.01 M phosphate buffer (pH 7.0) and 5 mM tetramethyl ammonium perchlorate. The flow rate was 0.8 ml/min with a column pressure of 2210 p.s.i. at ambient temperature. In this system, retention times for AZT and AZT-P^- were 8.86 and 3.19 min, respectively. For detection of AZT-aCDS the same system was used but the mobile phase consisted of 50% acetonitrile and 50% buffer. The retention time was 4.7 min. The limit of detection was 0.05 μg/g tissue.

2.4. In vitro stability studies

2.4.1. In vitro stability of AZT-P^- (3) to phosphodiesterase IV and alkaline phosphatase I

Control samples: 3 ml of buffer (pH 7.4 phosphate buffer, pH 8.8 Tris buffer, pH 9.8 glycine buffer) and 60 μl of stock solution of AZT-P^- (1.0 mg/ml) were measured into a vial. The vial was closed by a cap and put into a waterbath at a temperature of 37°C. At appropriate time intervals aliquots (400 μl) were removed and added to 800 μl of acetonitrile containing 5% DMSO and 1% acetic acid. Twenty μl of this solution were analyzed by HPLC. Enzyme-treated samples: The above buffer solutions were used with addition of 3 mg type IV phosphodiesterase I or 60 μl alkaline phosphatase.

2.4.2. In vitro stability of AZT-aCDS (4)

Adult, male, New Zealand, white rabbits weighing 2500–3000 g were used. The animals were kept in individual cages with free access to food and water. A group of five rabbits was used, and another group of three animals served as control. In vitro investigations were performed in blood, brain, liver, lung, and testes, respectively. Freshly collected whole blood was used. Tissue homogenates were prepared by homogenizing (using a Tekmar-tissuemizer) freshly collected organ tissues with isotonic phosphate buffer (pH 7.4) to give 20% (w/w) homogenate. Four hundred μl of stock solution of AZT-aCDS (4) (concentration, 1.0 mg/ml in DMSO) were added to 5 g of 20% (w/w) biological medium at 37°C and mixed using a Fisher-brand Touch-Mixer for 10 s. At appropriate time intervals, 400 μl of samples were taken and mixed to 800 μl of acetonitrile containing 5% DMSO and 1% acetic acid. The mixture was shaken using the Touch-Mixer for 1 min and centrifuged for 12 min at 12000 r.p.m. The supernatant was removed with an insulin syringe and filtered through a Millipore (Type H, pore size 0.45 μm) filter. The solution was analyzed by HPLC, injecting 20 μl of sample. Quantitation was done by a calibration curve.

2.5. In vivo distribution/metabolism studies

Groups of at least three rabbits were used. AZT-aCDS (4) was dissolved in DMSO and administered in the ear vein of conscious animals at a dose of 54.4 mg/kg. In the control group only the solvent was administered. Animals were sacrificed by injection of a pentobarbital overdose at appropriate time intervals (0.1, 3, 8, 24, 30 and 48 h). Trunk blood was collected into heparinized tubes. Organs (brain, liver, lung, and testes) were removed and immediately frozen. Samples for HPLC analysis were prepared as described previously for the in vitro studies.

3. Results and discussion

3.1. Synthesis

The synthetic route for the synthesis of the AZT anionic chemical delivery system AZT-aCDS (5′-(3′-azido-3′-deoxythymidyl)-hexanoyloxymethyl methylphosphonate), is summarized in Fig. 1. Chloromethyl hexanoate (1) was prepared by reaction of hexanoyl chloride with

paraformaldehyde in the presence of a catalytic amount of $ZnCl_2$ (Yoshimura et al., 1987). This compound was converted to iodomethyl–hexanoate (2) by reaction with NaI in dry acetone. This is one of the two components of the final condensation reaction. The other one, AZT-P$^-$ (3), was obtained from AZT with methylphosphonic dichloride in dry acetone. The target compound, AZT-aCDS (4), was formed from compounds (2) and (3) in dry DMF under nitrogen atmosphere, a modification of the reaction conditions of Clark and Miller (1977). The obtained AZT-aCDS is a slightly yellow viscous oil; it is stable at 4°C for more than 1 year if protected from light and moisture.

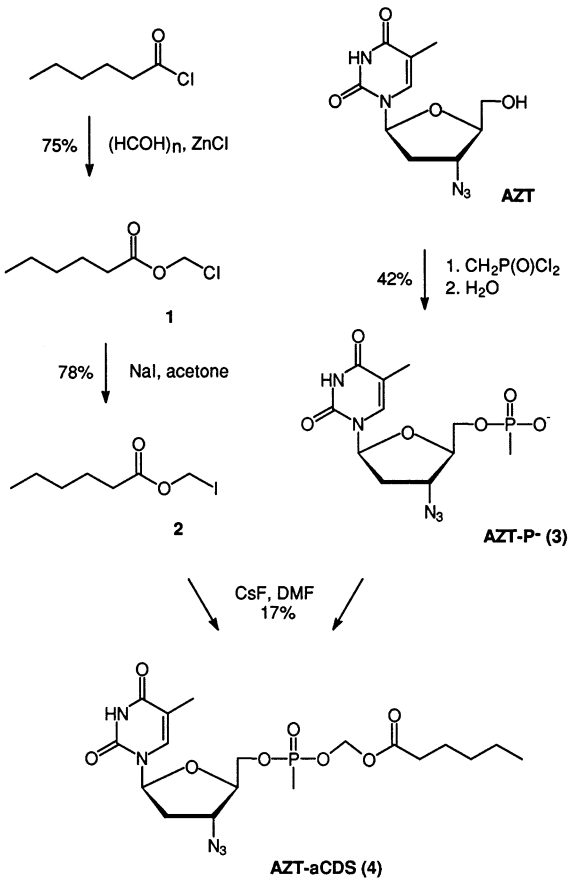

Fig. 1. Synthesis of the anionic chemical delivery system (aCDS) for AZT (zidovudine).

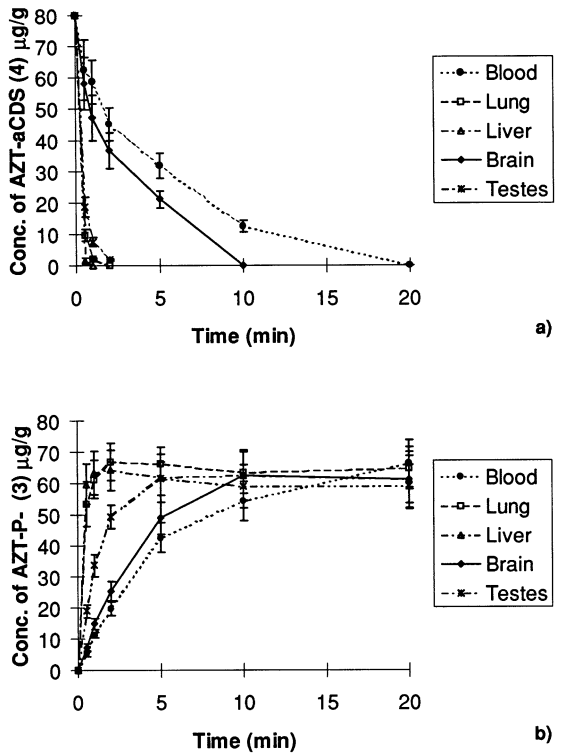

Fig. 2. In vitro concentration change for (a) AZT-aCDS (4) and (b) the negatively charged intermediate AZT-P$^-$ (3) in various organs.

3.2. In vitro stability studies

The in vitro stability of AZT-aCDS (4) was examined in different rabbit organs at 37°C, as shown in Fig. 2(a). Its concentration decrease was fairly rapid and, accordingly, AZT-aCDS levels fell under detection limits in 10 min, with the exception of blood samples. Here, AZT-aCDS could be detected until 20 min. Half-lives found in different organs are as follows: blood 3.81 min ($r = 0.9513$), lung 0.44 min ($r = 0.9949$), liver 0.17 min ($r = 0.9969$), brain 2.94 min ($r = 0.9601$), and testes 0.87 min ($r = 0.9844$) (Fig. 3). The data show that the degradation process is the fastest in liver but, even in the lung and testes, half-lives are less than 1 min. Together with the disappearance of AZT-aCDS, AZT-P$^-$ (3) appears as a main metabolite in increasing concentration (Fig. 2(b)). Within the investigated time intervals, AZT-release was not detectable. These results support the

hypothesis (Somogyi et al., 1997) that the main decomposition step of AZT-aCDS is the hydrolytic cleavage by esterases yielding AZT-P$^-$ (3) as a main metabolite. Cleavage of this less-hindered hexanoate ester function is faster than that of the previously employed pivaloate ester in testosterone-aCDS, proving that the metabolism of such aCDSs is controllable as required by the general principles of retrometabolic drug design (Bodor and Buchwald, 1997).

3.3. In vivo studies

The aCDS (4) was administered in the ear veins of conscious animals (three rabbits/group) at a dose of 54.4 mg/kg body weight. Since very rapid hydrolytic decomposition was found in the in vitro investigations, it was not surprising that, even at the first sampling point (6 min), the starting compound could not be detected. The AZT-P$^-$ (3) resulting from the rapid hydrolytic cleavage of AZT-aCDS reached its highest concentration in all organs within 6 min (Fig. 4). The concentration maximum is the lowest in blood (about 40 μg/g) and the highest, approximately four times higher, in lung. In testes, brain, and liver, the concentration maximum of AZT-P$^-$ was between 65 and 82 μg/g, respectively. In all sampled organs, with the exception of blood, the disappearance of AZT-P$^-$ is relatively slow. The blood concentration decreased to about 50% of its maximum within 3 h. After this period, the metabolite gradually disappeared, and the concentration level fell under the detection limit (0.05 μg/g) at 48 h. In brain, liver, lung, and testes, AZT-P$^-$ levels did not change significantly in the first 24 h, then disappeared around 48 h.

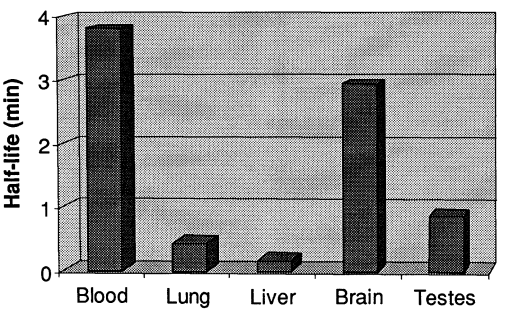

Fig. 3. In vitro half-lives of AZT-aCDS (4) in different organs. Each value is the mean of five independent determinations.

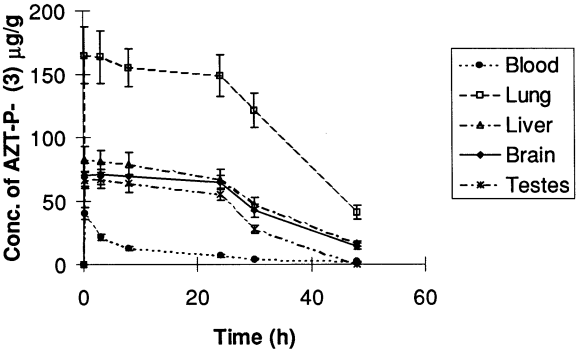

Fig. 4. In vivo concentration change for the negatively charged intermediate AZT-P$^-$ (3) in various organs after i.v. administration of a 54.4 mg/kg AZT-aCDS dose in rabbit. Each value is the mean of three independent determinations. Due to rapid metabolism, the starting material (AZT-aCDS) could not be detected in organ samples. Simultaneously with the disappearance of AZT-P$^-$, AZT could be detected in very low concentrations (not shown).

The delayed presence of the anion in the brain and its presence at levels higher than in blood is reassuring; it proves that the 'lock-in' mechanism works despite fears that active efflux pumps for organic anions might remove these negatively charged intermediates. The existence of probenicid-sensitive, active pumps for organic anions such as beta-lactam drugs or valproic acid (Cornford et al., 1985; Adkinson et al., 1994) has been long suspected (Barza, 1993), and evidence for their existence is accumulating (Deguchi et al., 1997; Suzuki et al., 1997). There is also recent in vivo evidence that AZT is recognized and actively transported out of the brain by a probenicid-sensitive efflux system (Dykstra et al., 1993; Wong et al., 1993); the corresponding organic anion carrier may be located at the blood–CSF barrier (choroid plexus) (Masereeuw et al., 1994). Simultaneously with the disappearance of AZT-P$^-$, AZT could be detected, albeit in very low concentrations. In the brain, it reached a maximum concentration of $\sim$0.1 μg/g, about two to three times the detection limit, but below virustatic levels ($\sim$0.3 μg/g). However, this level was maintained for a relatively long time, whereas brain

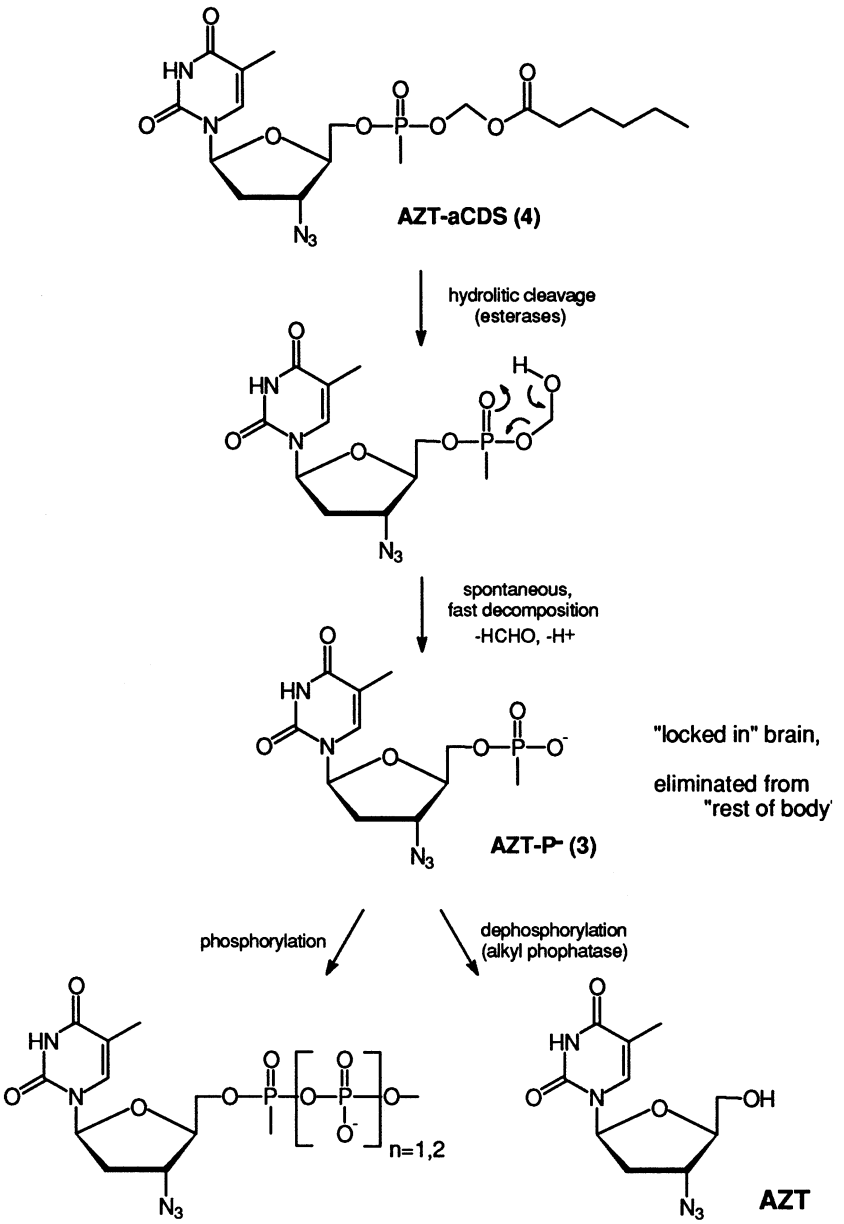

Fig. 5. Designed sequential metabolic conversion of the AZT-aCDS based on an (acyloxy)alkyl phosphonate derivative. This is a concrete example of the general system described earlier (Somogyi et al., 1997).

AZT levels after administration of AZT itself in rats (0.136 mmol/kg) disappeared completely after 1 h (Little et al., 1990). The metabolic sequence of this AZT anionic chemical delivery system is summarized in Fig. 5; it is a concrete example of the general sequential conversion designed for such aCDSs (Somogyi et al., 1997).

To study the process of phosphorolytic cleavage of the P–O bond, we investigated the action of pure enzymes (alkaline phosphatase, phosphodiesterase) at various pH values. With alkaline phosphatase at pH 7.4 and 9.8, AZT release could

not be detected in the first 20 h. Afterwards, some AZT was detected, but concentrations were very low: 0.61 and 0.28 μg/g at pH 7.4 and 9.8, respectively. However, at a pH value of 8.8 the phosphorolytic cleavage of this P–O bond occurs at a well measurable rate (Fig. 6). AZT-P$^-$ concentration decreased about 50% in a period of 136 h and, at the same time, the concentration of AZT increased continuously and reached a 7.06 μg/g final value. In the control samples, AZT could also be detected, but the concentration was found to be very low; the highest measured AZT concentration in control samples was only 1.03% of the value measured in the enzymatic sample. In the meantime, this phosphonate derivative of a primary hydroxyl group was rather resistant to phosphodiesterase attack; we were unable to detect AZT by incubation of AZT-P$^-$ and phosphodiesterase at various pH values (7.4, 8.8, 9.8). In a similar manner, phosphodiesterase I and II did not hydrolyze the α,β-methylene 5′-phosphonate derivative of AzddTDP within 24 h (Balzarini et al., 1988).

4. Conclusion

The novel, anionic chemical delivery system (aCDS) approach that uses an (acyloxy)alkyl phosphonate-type targetor moiety and was designed using the general principles of retrometabolic drug design, has been applied for brain-targeted delivery of AZT. For this system, the designed enzymatic sequence was completed. Hydrolytic cleavage of the hexanoate ester function, the first metabolic step, is faster than that of the previously employed, more hindered pivaloate ester, proving that the metabolism of such aCDSs is, indeed, controllable. While the phosphonate derivative of a secondary hydroxyl group proved resistant to phosphorolytic attack, the second metabolic step that releases the active drug, alkaline phosphatase cleaves the P–O bond of the phosphonate derivative of the primary hydroxyl group in this system, but only at a relatively slow rate. Consequently, some AZT released from systemically administered aCDS was detectable in the brain of rabbits but only at very low concentrations; recently discovered, active efflux pumps for AZT might have had a role in this. As a general conclusion, even if the present aCDSs could not deliver sufficiently high drug levels, the completion of the designed metabolic sequence and the controllable nature of the metabolic steps were proven. Since there is enough flexibility in the model, further, adequate manipulations might render such anionic chemical delivery systems into a useful addition of the drug targeting arsenal.

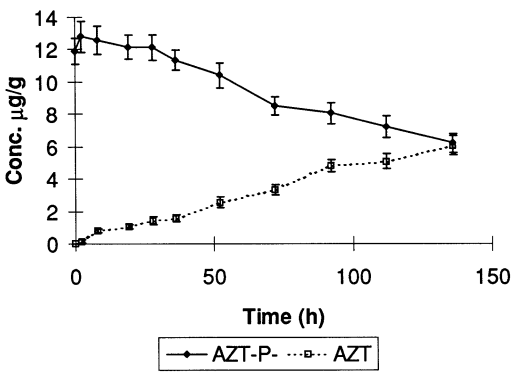

Fig. 6. In vitro enzymatic assay of TP$^-$ with alkaline phosphatase at pH 8.8. In control samples some AZT could also be detected, but the highest detected concentration was only 1.03% of the value measured in the enzymatic sample. No significant degradation was detected with phosphodiesterase (pH 7.4, 8.8, 9.8; not shown).

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