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Metabolic properties of phosphonate esters

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The object of the present work was to investigate the difference in the metabolism of the phosphonate derivatives of primary or secondary hydroxyl groups. To study the phosphorolytic cleavage of such P–O bonds, zidovudine (AZT) hexanoyloxymethyl-methylphosphonate (HOM-AZT-P), an ester of a primary OH functionality, and methyl-pivaloyloxymethyl-testosterylphosphonate (POM-T-P), an ester of a secondary OH functionality, were prepared. The actions of pure enzymes such as alkaline phosphatase and phosphodiesterase on the corresponding phosphonate compounds (AZT-P and T-P) were investigated at various pH values. The phosphonate derivative of the secondary hydroxyl group of testosterone proved completely resistant to such phosphorolytic attacks, and release of free testosterone could not be detected. The phosphonate derivative of the primary hydroxyl group of zidovudine proved resistant to phosphodiesterase, but not to alkaline phosphatase, and in this second case, release of free zidovudine could be detected.

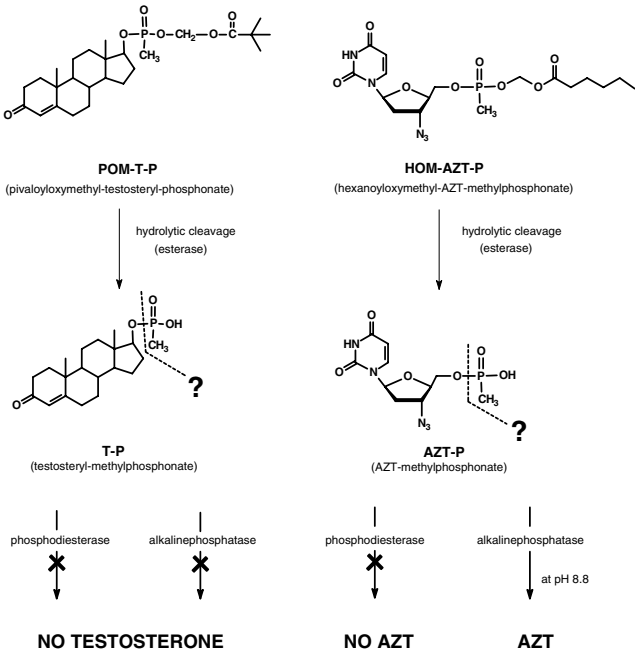
1. Introduction

Relatively few medicinal chemistry and/or pharmaceutical publications deal with the examination of phosphonate and phosphate analogs. There have been a few reports on the advantageous properties of (acyloxy)alkyl derivatives of organic phosphate, which increase penetration across biological membranes (Farquhar et al. 1983; Sastry et al. 1992; Srivastva and Farquhar 1984). An (acyloxy)alkyl ester of phosphonoformate was also prepared, and (acyloxy)alkyl prodrugs have been used to improve the bioavailability of phosphonates (Iyer et al. 1989; Krapcho et al. 1988). (Acyloxy)alkyl, glycolamide-alkyl esters, or as the case may be, amides of carbonic acids were used in much wider circles (Bundgaard 1991).

In 1986, attention was drawn to phosphonate nucleosides as new compounds with antiviral effects (De Clercq et al. 1986). It was discovered that both purine and pyrimidine bases of cyclic nucleoside phosphonate analogs have wide-spectrum antiviral activity against certain RNA and DNA viruses (De Clercq 1991). Over the past few years, numerous esters of nucleoside phosphonates, or as the case may be, phosphates were synthesized and their metabolic properties were studied using *in vitro* and *in vivo* experiments. Mono- and bis-alkyl esters, as well as amides and mixed ester-amide compounds were produced in both cyclic and acyclic variations. A common characteristic of these compounds was that the phosphonate or phosphate ester itself, or their di- or tri-phosphate derivative was the active substance. The general conclusion reached during the examination of these compounds (Starrett et al. 1994) was that the simple alkyl esters or amides were not suitable as derivatives due to their weak stability and/or low biological activity.

However, (acyloxy)alkyl esters provide special "camouflaging" possibilities, and bis(pivaloyloxymethyl) (POM) and hexanoyloxymethyl (HOM) ester derivatives show particularly good activity and penetration properties (Starrett et al. 1992). The metabolic properties of these compounds have been examined, and it has been found that the bis-ester can transform into the parent compound in a hydrolytic process through the production of a mono-ester. Several *in vitro* and *in vivo* studies were performed on the antiviral activity of 9-(2-phosphonyl-methoxyethyl)adenine (PMEA) POM ester. The ester was found to hydrolyze within the cell releasing PMEA, and the formed charged phosphonate moiety inhibits the compound from leaving the cell quickly (Starrett et al. 1992). In this study, the authors were not concerned with the examination of the hydrolytic mechanism; therefore, it is unclear how many steps does the transformation require or whether the hydrolysis is chemical and/or enzymatic. Examination of tritiated bis-POM-PMEA, revealed that the production of the mono-ester primarily occurs extracellularly (Srinivas et al. 1993). In a cell-free environment, the bis-POM-PMEA hydrolyzed with an approximately 4-hour half-life into a mono-POM compound. Further transformation of the mono-compound into PMEA only occurred in the presence of cells or serum. The formed PMEA – in the presence of cells – was further transformed into the monophosphate and the biologically active diphosphate. These results demonstrate that the bis-POM-PMEA is comparatively unstable at biological pH, and transforms into a mono-POM compound in the absence of cells or serum,

Scheme



Metabolic conversions of POM-T-P and HOM-AZT-P

and only in the presence of these will hydrolyze in a second step into the PMEA compound. It was also verified that the bis-POM-PMEA is comparatively stable at pH 2, and its estimated half-life is approximately 24 hours (Starrett et al. 1992).

In cell homogenates or in metabolic examinations performed with purified carboxylesterase enzyme, transformation of bis-POM-PMEA was fast, and formation of mono-POM-PMEA was faster than expected on the basis of data regarding its chemical transformation. Hence, it was inferred that bis-POM-PMEA undergoes enzyme-catalyzed hydrolysis (the enzyme is most likely the esterase) (Annaert et al. 1997).

The role of the esterase was examined by incubation of bis-POM-PMEA and purified carboxylesterase. A fast hydrolysis of bis-ester into mono-ester occurred followed by the slow development of PMEA. For a high initial concentration of bis-POM-PMEA (100 M), the mono-ester was detected in the cell after 3 h, whereas for a lower initial concentration (50 μM), the compound was detected even earlier. This indicated that the phosphodiesterase responsible for formation of the mono-ester PMEA becomes saturated (Farquhar et al. 1994).

The enzymatic degradation of the bis-POM ester of 2′-deoxy-5-fluorouridin-5′-monophosphate was also examined (Farquhar et al. 1994). Stability testing found minimal degradation ($t_{1/2} > 100$ h) at pH 1 and 4 in a wet environment at room temperature. The compound was also relatively stable at pH 7.4 and 9, but it degraded quickly ($t_{1/2} < 2$ min) in 0.05 M NaOH. A degradation half-life of 4 h was found when the compound was incubated for 24 h at 37 °C with 0.3 unit carboxylesterase in a pH 7.4 phosphate buffer. If 1.0 unit of enzyme was used, the half-life decreased to 1.9 h, and if 2.5 units of enzyme were used, it decreased to 0.25 h. The product of the degradation was the mono-POM ester, and no other product was found during a 7 h incubation time (after 24 h, a substance without the POM group was detected in concentrations no exceeding 1–3%). Tests performed using ten-fold excess of alkaline phosphate, 5′-nucleotidase, or, as the case may be, phosphodiesterase I enzymes (37 °C, 2 h incubation time, 0.1 M Tris buffer, pH 8.0) demonstrated that the compound is not a substrate for such nucleotide-catabolyzing enzymes.

Such (acyloxy)alkyl-phosphonate ester derivatives of hydroxyl-containing drugs can be used to provide brain-targeted delivery, an important goal of many drug development projects (Bodor and Buchwald 2003a). Due to their increased lipophilicity, the esters can by-pass biological membranes, including the blood-brain barrier, by passive transport. A first hydrolytic cleavage by esterases releases a charged, hydrophilic phosphonate compound which can no longer by-pass the same membrane and, therefore, can provide sustained, site-specific release of the active drug following a phosphorolytic attack by alkaline phosphatase or by phosphodiesterase. The utility of chemical delivery systems that rely on similar targeting mechanism has been demonstrated in many cases (Bodor and Buchwald 1999; 2002; 2003b).

The resistance of P−O bonds towards phosphorolytic attacks should be different in more hindered secondary hydroxyl group-substituted phosphonate esters and in less hindered primary hydroxyl group-substituted phosphonate esters. The object of the present work was to investigate the difference in the metabolism of primary or secondary hydroxyl group-substituted phosphonate derivatives that are intended to provide brain-targeted delivery.

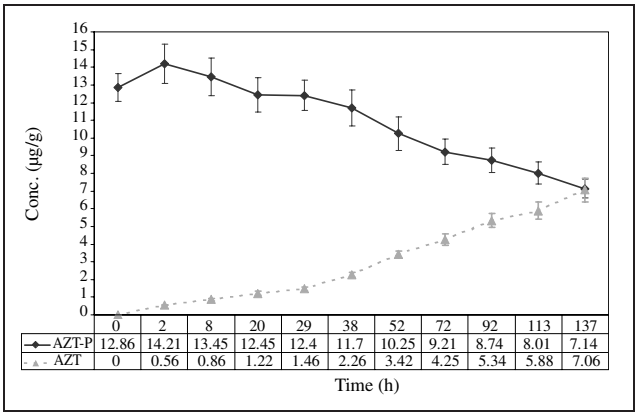
To study the phosphorolytic cleavage of such P−O bonds, zidovudine (AZT) hexanoyloxymethyl methylphosphonate (HOM-AZT-P), an ester of a primary OH group (Somogyi et al. 1998a), and methyl-pivaloyloxymethyl-testosteryl phosphonate (POM-T-P), an ester of a secondary OH group (Somogyi et al. 2002; Somogyi et al. 1998b), were prepared. The actions of pure enzymes such as alkaline phosphatase and phosphodiesterase on the corresponding phosphonate compounds (AZT-P and T-P; Scheme) were investigated at various pH values.

2. Investigations and results

2.1. In vitro study of AZT-P with phosphodiesterase IV and alkaline phosphatase I

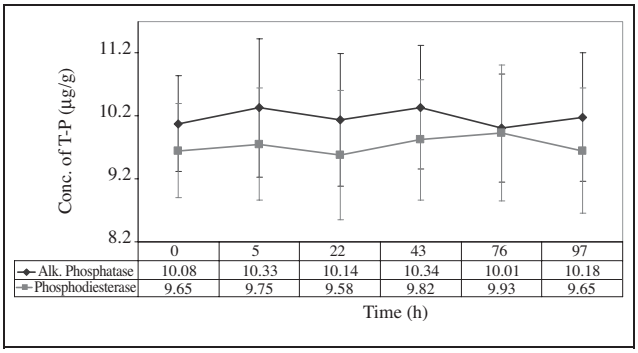
Buffer solutions (at various pH values) and stock solution of AZT-P were put into a water-bath. For enzyme-treated samples, type IV phosphodiesterase or type I alkaline phosphatase was added to this buffer solution. At appropriate time intervals, aliquots were removed and analyzed by HPLC.

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 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 occurred at a well measurable rate (Fig. 1). AZT-P concentration decreased about 50% within a period of 136 h, and, at the same time, the concentration of AZT increased continuously and reached a final value of 7.06 μg/g. 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: AZT was undetectable following



	0	2	8	20	29	38	52	72	92	113	137
AZT-P	12.86	14.21	13.45	12.45	12.4	11.7	10.25	9.21	8.74	8.01	7.14
AZT	0	0.56	0.86	1.22	1.46	2.26	3.42	4.25	5.34	5.88	7.06

Fig. 1: *In vitro* enzymatic assay of AZT-P with alkaline phosphatase at pH 8.8



	0	5	22	43	76	97
Alk. Phosphatase	10.08	10.33	10.14	10.34	10.01	10.18
Phosphodiesterase	9.65	9.75	9.58	9.82	9.93	9.65

Fig. 2: *In vitro* enzymatic assay of T-P with alkaline phosphatase and phosphodiesterase (released testosterone could not be detected)

incubation of AZT-P and phosphodiesterase at various pH values (7.4, 8.8, and 9.8).

2.2. In vitro study of T-P with phosphodiesterase IV and alkaline phosphatase I

Buffer solutions (at various pH values) and stock solution of T-P were put into a water-bath. For enzyme-treated samples, type IV phosphodiesterase or type I alkaline phosphatase was added to this buffer solution. At appropriate time intervals, aliquots were removed and analyzed by HPLC.
The phosphonate derivative of the secondary hydroxyl group in T-P was found to be fairly resistant to phosphorolytic attack by either alkaline phosphatase or phosphodiesterase. Released testosterone could not be detected in any of the cases (Fig. 2).

3. Discussion

The metabolic conversions of POM-T-P and HOM-AZT-P are summarized in the Scheme. The phosphonate derivative of the more hindered secondary hydroxyl group was resistant to both alkaline phosphatase and phosphodiesterase. Free testosterone could not be detected following incubation of T-P with either phosphodiesterase or alkaline phosphatase at various pH values (7.4, 8.8, and 9.8).
Whereas the phosphonate derivative of a secondary hydroxyl group proved resistant to phosphorolytic attacks, the phosphonate derivative of a primary hydroxyl group was cleaved by alkaline phosphatase at a pH value of 8.8 with a well-measurable rate. Free AZT could be detected with the highest concentration measured at the end of the incubation time (136 h). Nevertheless, phosphodiesterase was unable to cleave the P−O bond of the phosphonate derivative of this primary hydroxyl group, and free AZT could not be detected during the incubation time if phosphodiesterase was used.

4. Experimental

4.1. Analysis

A HPLC method was developed for the quantitative analysis of the different compounds and their metabolites. 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, and SP 4290 integrator.
Parameters for AZT-P:Column: Supelco LC-8-DB, 3 μ (75 × 4.5 mm I.D.) with a 15 mm (3 μ) LC-8-DB guard. Mobile phase: 10% of AcCN, 90 % of 0.01 M phosphate buffer (pH 7.0), and 5 mM tetramethyl ammonium perchlorate. Flow rate: 0.8 ml/min at ambient temperature. Wavelength: 266 nm. Retention times: 8.86 min for AZT, 3.19 min for AZT-P. The limit of detection was 0.05 μg/g.
Parameters for T-P: Column: Bondclone C18 (Phenomenex, Torrence, CA) (150 × 3.9 mm I.D.) with a 15 mm C18 guard (Rainin, Ridgefield, NJ). Mobile phase: 50% AcCN, 50% of 0.01 M phosphate buffer (pH 7.0). Flow rate: 0.9 ml/min at ambient temperature. Wavelength: 254 nm. Retention times: 9.06 min for testosterone, 4.88 min for T-P. The limit of detection was 0.05 μg/g.

4.2. In vitro study

AZT-P: Control samples: 3 ml of buffer (pH 7.4 phosphate buffer, pH 8.8 Tris buffer, pH 9.8 glycine buffer) and 6 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 water-bath 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. 20 μl of this solution was analyzed by HPLC.
Enzyme-treated samples: The above buffer solution was used with addition of 3 mg of phosphodiesterase IV or 60 μl of alkaline phosphatase I.
T-P: Control samples: 3 ml of pH 8.8 Tris buffer or pH 7.4 phosphate buffer and 60 μl of stock solution of T-P (1.0 mg/ml) were measured into a threaded 20 ml vial, which was then closed by a teflon-lined, phenolic cap and was put into a water-bath. The reaction was performed at 37 °C, and at appropriate time-intervals, aliquots (400 μl) were removed and added to 800 μl of acetonitrile containing 5 % DMSO and 1% acetic acid. Of this solution, 20 μl was analyzed by HPLC. For the enzyme-treated sample the above buffer solutions were used with the addition of 3 mg of phosphodiesterase IV. The procedure was the same for alkaline phosphatase, but in this case pH 9.8 glycine buffer and 60 μl enzyme were used.

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