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Targeted drug delivery to the brain via phosphonate derivatives I. Design, synthesis and evaluation of an anionic chemical delivery system for testosterone

Gabor Somogyi [1], Sinji Nishitani [2], Daishuke Nomi, Peter Buchwald, Laszlo Prokai, Nicholas Bodor *

Center for Drug Discovery, University of Florida, Health Science Center, PO Box 100497, Gainesville, FL 32610-0497, USA

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Abstract

An anionic chemical delivery system (aCDS) was designed and evaluated for brain-targeted delivery of testosterone (T). In this system, targeting is achieved through the use of a specific, (acyloxy)alkyl-phosphonate-type, targetor moiety. The systemically administered T-aCDS can enter the brain by passive transport due to its increased lipophilicity. Hydrolytic cleavage by esterases releases, via a chemically unstable, short-lived intermediate, a negatively charged, hydrophilic phosphonate compound (TP^-). This is locked in the brain and should provide sustained, site-specific release of the active drug following a phosphorolytic attack by alkaline phosphatase or by phosphodiesterase. In vivo evaluations found maximum T-aCDS brain levels 5–10 min after administration; they fell under the detection-limit (< 0.1 μg/g) after 60 min. With the employed (pivaloyloxy)methyl phosphonate ester, cleavage by esterases, the first metabolic step in the decomposition process, was not very fast. Maximum concentration of the decomposition product (TP^-) was obtained at 30 min after administration; it did not decrease significantly during the study proving that this negatively charged intermediate is 'locked in' the brain. However, the phosphonate derivative of the secondary, hindered hydroxyl group in this product was fairly resistant to phosphorolytic attack, the second metabolic step. The released drug could not be detected indicating that testosterone release, if any, is slower than metabolism and/or elimination.

Keywords: Brain-targeted delivery; Blood-brain barrier; Anionic chemical delivery system; Testosterone; Phosphodiesterase; Alkaline phosphatase

* Corresponding author. Tel.: +1 352 3928186; fax: +1 352 3928589.
[1] On leave of absence from the Department of Forensic Medicine of the Medical University of Debrecen, Hungary
[2] S. Nishitani and D. Nomi are on leave of absence from the Otsuka Pharmaceutical Company, Ltd., Tokushima, Japan.

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1. Introduction

The aim of the present work was to develop and investigate targeted drug delivery via phosphonate derivatives. The basic concept is the same as for all other enzymatic physical–chemical based chemical delivery systems (CDS): to achieve targeting by exploiting site specific transport properties like those of the blood–brain barrier (Bodor and Brewster, 1983, 1991; Bodor, 1987, 1992, 1995b). With this approach, the drug is chemically modified to introduce the targetor moiety and the eventual protective function(s). Upon administration, the resulting CDS is distributed throughout the body. Predictable enzymatic reactions convert the original CDS by removing some of the protective functions and modifying the targetor moiety, leading to an intermediate form that has significantly different physicochemical properties. While these intermediates are continuously eliminated from the 'rest of the body', the specific transport properties of the blood–brain barrier—which can be regarded as a biological membrane that is permeable to most lipophilic compounds but not to hydrophilic molecules—will provide a specific concentration in the brain. If a lipophilic CDS that can enter the brain is converted there to a hydrophilic molecule, one can assume that it will be 'locked-in': it will no longer be able to come out, ultimately allowing release of the active drug only at the site of action. This concept was already successfully applied to a variety of drugs using a 1,4-dihydrotrigonelline↔trigonelline redox system where the 'locked-in' intermediate is the drug-quaternary targetor (DQ^+) cation (Bodor and Brewster, 1991; Bodor, 1995b; Bodor and Buchwald, 1997).

The present work explores the possibilities of a novel approach, which we designated as anionic chemical delivery system (aCDS). Here, an (acyloxy)alkyl-phosphonate type targetor moiety is used, and formation of an anionic intermediate is expected to provide the 'lock-in' (Bodor, 1995a). The biologically active compound is coupled to this targetor moiety to obtain the original, neutral form of the aCDS that should be lipophilic enough to penetrate the blood–brain barrier (BBB) via passive diffusion and enter the target organ after systemic administration. Hydrolytic cleavage by esterases releases a negatively charged, hydrophilic phosphonate compound via a chemically unstable, short-lived intermediate that spontaneously decomposes by cleavage of an aldehyde-type unit (Farquhar et al., 1983). The anionic phosphonate is locked in the brain and should provide sustained, site-specific release of the active drug following a phosphorolytic attack by alkaline phosphatase or by phosphodiesterase. In the meantime, conversion of the original, lipophilic form to the hydrophilic intermediate that also takes place in the 'rest of the body' after overall distribution, actually accelerates peripheral elimination and further contributes to brain targeting. The mechanism is summarized in Fig. 1. Selection of adequate protective groups should ensure the specific sequence of these designed enzymatic conversions; these groups should also be compatible with their biological environment to avoid toxic effects. Possible substitutions at the R_1, R_2, R_3 positions (Fig. 1) should render sufficient flexibility to the system to overcome eventual problems related to distribution or rate of metabolism.

Here we report studies on the design, synthesis, and organ-targeting properties of such a novel aCDS for testosterone (T,1). Selection of testosterone was motivated by our earlier work on the delivery of testosterone using a redox chemical delivery system (Bodor and Farag, 1984) where the reasons for brain-targeting of this hormone have been also summarized. For such lipophilic compounds, the main goal of a CDS approach is not as much to enhance BBB penetration, but to provide long-lasting, elevated brain concentrations by this special sequestering mechanism. For example, estradiol (E_2) released from the E_2Q^+ intermediate that is formed after E_2-CDS administration in rats has an elimination half-life of more than 200 h (Mullersman et al., 1988), and E_2-levels in brain tissue after E_2-CDS administration are elevated four to five times longer than after simple E_2 treatment (Sarkar et al., 1989; Bodor and Brewster, 1991).

The major novelty of the present approach is the use of an (acyloxy)alkyl-phosphonate moiety, in the present case a (pivaloyloxy)methyl (POM)

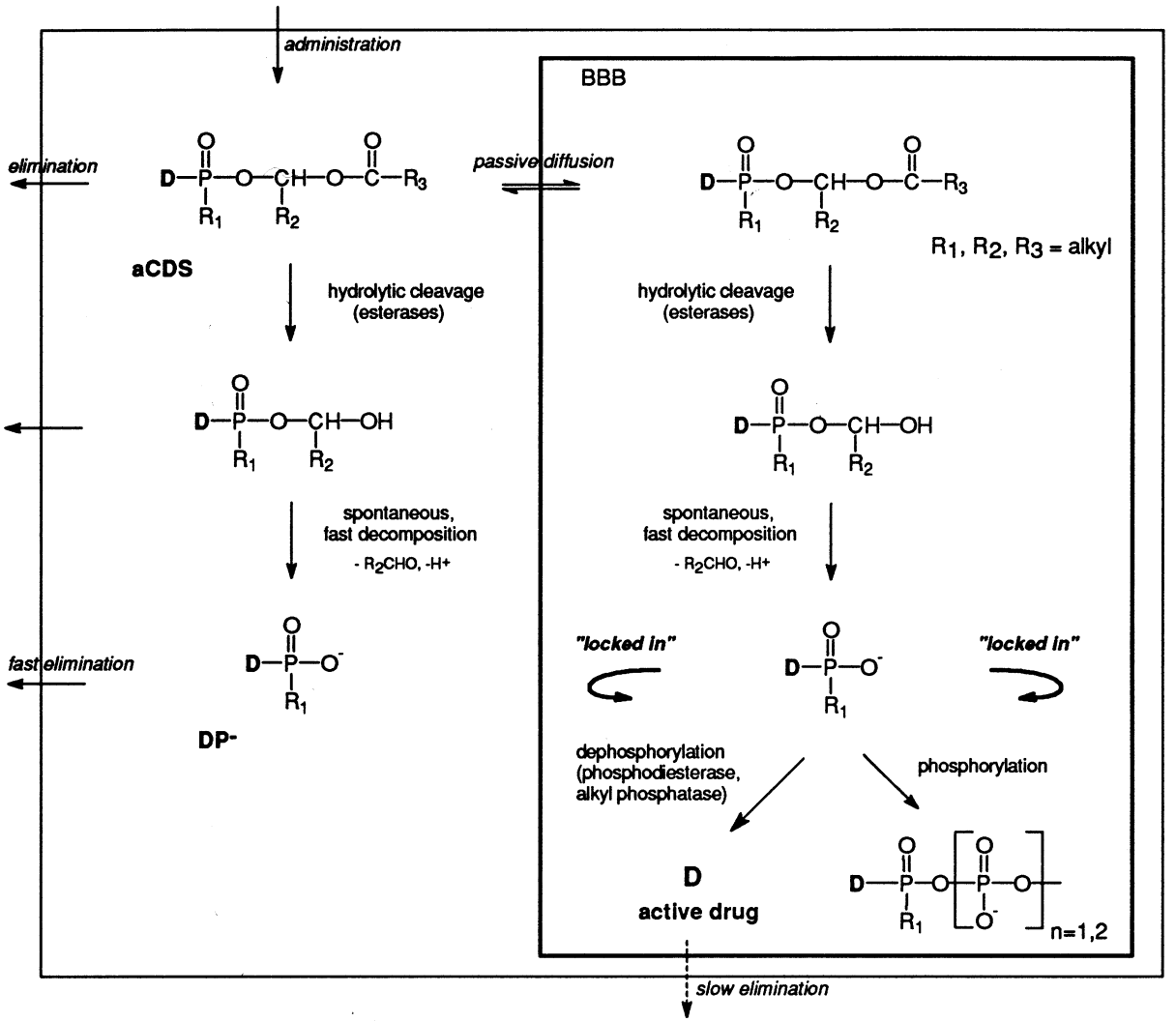

Fig. 1. Designed sequential metabolic conversion of the anionic chemical delivery system (aCDS) based on (acyloxy)alkyl-phosphonate derivatives. The original, lipophilic system is distributed through the body and can by-pass the BBB. Hydrolytic cleavage by esterases releases, via a chemically unstable, short-lived intermediate, a negatively charged, hydrophilic phosphonate compound (DP^-). This is locked in the brain and should provide sustained, site-specific release of the active drug (D) following a phosphorolytic attack by alkaline phosphatase or by phosphodiesterase. Phosphorylation of DP^- is another possible, well-known metabolic reaction.

phosphonate moiety for targeting purposes. While for carboxylic acids a wide range of derivatives has been prepared and investigated, and, since their introduction by Jansen and Russell (1965), even (acyloxy)alkyl-type double esters have been extensively used (Bundgaard, 1985), there are only relatively few publications dealing with derivatives of phosphonates or their closely related analogues. Nevertheless, after the introduction of the concept by Farquhar et al. (1983), a number of studies report the use of some (acyloxy)alkyl derivatives of organophosphates as lipophilic prodrugs to enhance penetration across biological membranes. However, in all these studies the phosphonate moieties are not attached to the active drug, rather the phosphonate or phosphate

esters themselves, or their di- and triphosphate derivatives, are an integral part of the active component as illustrated by cAMP (Schultz et al., 1993), by a phosphonate-containing insulin receptor tyrosine kinase inhibitor (Saperstein et al., 1989), or by antiviral agents like PMEA (De Clercq, 1991; Starrett et al., 1992, 1994; Annaert et al., 1997), FdUMP (Farquhar et al., 1994), ddUMP (Sastry et al., 1992), etc. (Srivastva and Farquhar, 1984; Srinivas et al., 1993).

The (acyloxy)alkyl ester of phosphonoformate was also prepared (Iyer et al., 1989), and (acyloxy)alkyl prodrugs have been used to improve the bioavailability of phosphinates (Krapcho et al., 1988). Since their discovery (De Clercq et al., 1986), much attention was focused on phosphonate nucleosides as broad-spectrum antiviral agents (De Clercq, 1991), especially on the adenine analogue 9-(2-(phosphonomethoxy)ethyl) adenine (PMEA). Masking of the phosphonate functionality was expected to increase membrane permeability and oral bioavailability, and different mono or bis-alkyl ester, alkyl amide, and (acyloxy)alkyl phosphonate prodrugs were prepared and evaluated (Starrett et al., 1992, 1994). In general, it was found that simple alkyl ester or amide derivatives cannot be successfully used because of poor stability and/or poor activity. (Acyloxy)alkyl esters of PMEA served, however, as a unique mask for the phosphonate group and indeed provided remarkable enhancement of permeation across biological membranes and of oral bioavailability. The metabolism of the bis-(pivaloyloxy)methyl (POM) ester of PMEA was studied both in vitro and in vivo, and partial enzymatic hydrolysis was observed.

Based on such information, a (pivaloyloxy)methyl (POM) phosphonate ester derivative was selected to use as targetor moiety in a first evaluation of an anionic chemical delivery system. However, as negatively charged compounds tend to be poor substrates to carboxylate esterase (Krisch, 1971), we avoided the bis-esters where the negatively charged mono-ester that forms after the first hydrolytic cleavage is likely to resist further decomposition. Several observations confirm this hypothesis. For tritiated bis(-POM)PMEA, it was found that it is hydrolyzed primarily to the mono(POM)PMEA derivative ($t_{1/2}$ ~ 4 h) in cell-free medium, but further break down to PMEA takes place only in the presence of cells or serum, and phosphodiesterase, not carboxylesterase, is thought to be responsible for the conversion of mono(POM) ester into free PMEA (Starrett et al., 1992). The study also indicated that at high initial concentration of bis(POM)PMEA, there may be saturation of the phosphodiesterase. The role of the esterase was also studied by incubation of purified carboxylesterase with bis(POM)PMEA (Annaert et al., 1997) and with bis(POM)FdUMP (2′-deoxy-5-fluorouridine 5′-monophosphate) (Farquhar et al., 1994). In both cases, hydrolysis of the bis-ester into mono-ester was fast, and it was eventually followed by very slow formation of parent compound. Again, the second step most likely involves not carboxylesterase but phosphodiesterase, an enzyme that can catalyze the hydrolysis of a wide range of synthetic phosphoesters, in addition to naturally occurring nucleotide substrates (Landt et al., 1980). This enzyme system (nucleotide pyrophosphatase/phosphodiesterase) is abundantly present in mammalian tissues (Haugen and Skrede, 1977). As in the present approach the phosphonate moiety is not part of the active drug but is attached to it at a hydroxyl group, the bis-esters, necessary for complete lipophilization of phosphonate compounds, were not even needed. Here we investigated a compound where attachment of the (acyloxy)alkylphosphonate anionic targetor was carried out at a secondary, relatively hindered, hydroxyl group; however, it is worth noting that, eventually with some modification, e.g. inclusion of a bridging group, attachments can also be carried out at mercapto, carboxyl, amino, amide, imide, or other functional groups (Bodor, 1995a). Furthermore, instead of the simple methyl-phosphonate used here, other phosphonates that might have better lipophilicity or phosphatase-substrate characteristics can also be employed.

A neutral (pivaloyloxy)methyl (POM) ester should provide enough lipophilicity for passive BBB transfer. Indeed, a recent study found that mono and bis POM-attachment significantly increased the log octanol–water partition/distribu-

tion coefficient (log P) of PMEA (Annaert et al., 1997). In the same work, very high intracellular PMEA concentrations were found, suggesting that the negative charge of PMEA trapped this compound inside the cells in a manner that resembles the mechanism used in our aCDS. Intracellular enzymatic activity should be therefore high enough to allow 'lock-in' not only behind the blood–brain barrier, but also inside the cells, which would be useful for intracellular delivery of, e.g. antiviral agents. Two additional observations also worth mentioning. Bis(POM)PMEA was found to be quite stable in acidic buffer; such derivatives are therefore likely to be also stable in a gastric environment when administered orally, and, indeed, in preliminary studies approximately 30% of the administered bis compound was found in circulation as PMEA after oral administration to monkeys (Srinivas et al., 1993). On the other hand, the aldehyde by-product of the first hydrolysis is somewhat worrysome with regards to toxicity, but extensive experience with (acetoxy)methyl esters of carboxylic acids revealed surprisingly little toxicity, at least in the short term (Schultz et al., 1993).

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, Milwaukee, WI. Acetone, benzene and DMF were dried with 4-Å molecular sieves. The originally dry pyridine was dried by refluxing with solid KOH, followed by fractional distillation (b.p. 115.6°C). Merck Kieselgel 60 (70-230 Mesh ASTM) and Aldrich Florisil (100–200 Mesh) were used for column chromatography. Phosphodiesterase I (EC.3.1.4.1.) type IV (0.028 U/mg solid), and alkaline phosphatase (EC. 3.1.3.1., 2000 U) were used for enzymatic assays and were purchased from Sigma, St. Louis, MO.

2.2. Synthesis

2.2.1. Methyl-17-testosterylphosphonate (TP^-, 2)

A solution of testosterone (T,1) (5 g, 17.4 mmol) in dry pyridine (17 ml) was added dropwise between −3 and 0°C over a 25-min period to a stirred solution of methylphosphonic dichloride, $CH_3P(O)Cl_2$ (4.6 g, 34.7 mmol) (Riess and Ourisson, 1965). The resultant mixture was stirred for 1 h at room temperature, then poured into ice water, neutralized with sodium bicarbonate, and extracted twice with 175 ml of ether. The combined ether extract was washed with 175 ml of saturated aqueous sodium bicarbonate solution. The aqueous layer was acidified with 4 N hydrochloric acid while cooling in an ice bath. The flask was stored in a refrigerator overnight. The white precipitate which formed was removed by filtration, washed with cold water, and dried under vacuum at 50–60°C. The yield was 4.84 g, 76%. Recrystallization from aqueous methanol gave crystals melting at 189–191°C. NMR ($CDCl_3$): 0.83 (s, 3 H), 1.18 (s, 3 H), 1.30 (d, 3 H, $J = 18$ Hz), 0.7–2.5 (m, 19 H), 4.15 (m, 1 H), 5.68 (s, 1 H), 7.93 (bs, 1 H). Elemental analysis for $C_{20}H_{31}O_4P$. Theory: C, 65.56; H, 8.53. Found: C, 65.61; H, 8.56.

2.2.2. Methyl-17-testosterylphosphonate silver salt (3)

The phosphoric acid derivative produced above (4.3 g, 11.7 mmol) was dissolved in 2 N aqueous sodium hydroxide solution (6.16 ml, 12.32 mmol). A few drops of phenolphtalein solution were added and the alkaline solution was neutralized with 2 N nitric acid until the red color due to phenolphtalein disappeared. Then, a solution of silver nitrate (2 g, 11.7 mmol) in 6 ml of water was added in the dark. The resultant mixture was

allowed to stand in the dark overnight. The precipitate which formed was removed by filtration and washed with cold water, also in the dark (Methoden der Organischen Chemie, 1964). The off-white material was dried in vacuum in the dark at 60–80°C, and used for the next reaction without further purification. The crude silver salt, was obtained in 98% yield (5.47 g).

2.2.3. Iodomethylpivalate (5)

Sodium iodide (24.73 g, 165 mmol) was added to a solution of chloromethyl pivalate (4) (5 g, 33 mmol) in dry acetone (40 ml). Acetone was dried with anhydrous potassium carbonate and then distilled (b.p. 52.6°C). The mixture was stirred for 4 h at room temperature. Insoluble materials were removed by filtration and washed with fresh acetone. The filtrate was evaporated, and hexane and 5% aqueous sodium thiosulfate solution were added to the residue. The mixture was thoroughly shaken, then the organic layer was separated and washed with 5% aqueous sodium thiosulfate solution. Drying over anhydrous sodium sulfate, followed by evaporation of the solvent, afforded 7.03 g (88% yield) of yellow liquid which was used for the synthesis of methylpivaloyloxymethyl-17-testosterylphosphonate (6) without further purification, NMR ($CDCl_3$): 1.18 (s, 9 H), 5.88 (s, 2 H).

2.2.4. Methyl-pivaloylexymethyl–17-testosterylphosphonate (T-aCDS, 6)

Crude iodomethyl-pivalate (160 mg, 0.66 mmol) was dissolved in 2 ml of benzene and washed successively with 5% aqueous sodium thiosulfate (1 ml) and water (3×1 ml) and dried over anhydrous sodium sulfate. This solution was added dropwise into a stirred suspension of the methyl-17-testosterylphosphonat silver salt (3) (250 mg, 0.53 mmol) in 5 ml of dry benzene under nitrogen, in the dark and over a 20-min period. The resultant mixture was stirred at room temperature overnight. Insoluble materials were removed by filtration and the filtrate was washed, once with sodium thiosulfate solution and three times with water, then dried over anhydrous magnesium sulfate. Evaporation of the solvent gave a yellowish, viscous oil. The crude product was purified by column chromatography on silica gel, using 1:1 hexane-ethyl acetate as eluent. A slightly yellow viscous oil was obtained in 31% yield (80 mg). MS and NMR data were consistent with the assigned structure. MS: m/z 503 $(M + Na) +$. NMR ($CDCl_3$): 0.81 (s, 3 H), 1.17 (s, 3 H), 1.50 (d, 3 H, $J = 18$ Hz), 0.70–2.50 (m, 19 H), 4.14 (m, 1 H), 5.60 (d, 2 H, $J = 14$ Hz), 5.67 (s, 1 H). Elemental analysis for $C_{26}H_{41}O_6P$: Theory: C, 64.98; H, 8.60. Found: C, 64.92; H, 8.63.

2.3. Analytical methods

A high-performance liquid chromatography (HPLC) method was developed for the assay (quantitative analysis) of the different compounds and their metabolites in biological fluids. 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 254 nm, and SP 4290 integrator. A 150×3.9 mm (I.D.) reverse phase Bondclone C18 column (Phenomenex, Torrance, CA), operated at ambient temperature, was used for all separations. The column was protected with a 15×3.2 mm (I.D.) C18 guard column (Rainin, Ridgefield, NJ) packed with 7 μm of packing material. The mobile phase consisted of 50% acetonitrile and 50% phosphate buffer (pH 7.0). At a flow rate of 0.9 ml/min, testosterone, methyl-17-testosterylphosphonate and methyl-pivaloyloxymethyl-17- testosterylphosphonate had retention times of 9.06, 4.88, and 7.74 min, respectively.

2.4. In vitro stability studies

2.4.1. In vitro stability of TP^- (2) to phosphodiesterase IV and alkaline phosphatase I

Control samples: 3 ml of pH 8.8, Tris buffer or pH 7.4 phosphate buffer and 60 μl of stock solution of the investigated compound (~ 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 waterbath. 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 type IV phosphodiesterase I. The procedure was the same for alkaline phosphatase but in this case pH 9.8 glycine buffer, and 60 μl enzyme were used.

2.4.2. In vitro stability of T-aCDS (6)

A group of seven rats was used, and another group of three animals served as control. In vitro investigations were performed in blood, brain, liver, and lung, 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. 400 μl of stock solution of investigated compound (~ 1.0 mg/ml in DMSO) was added to 5 g of 20% (w/w) biological medium at 37°C and was mixed using a Fisher-brand Touch-Mixer for 10 s. The vials were put back into the waterbath. At appropriate time intervals (0.5, 2, 5, 10, 20, 30, 60 min), 400 μl of samples were taken and mixed to 800 μl of acetonitrile containing 5% DMSO and 1% acetic acid in an Eppendorf microcentrifuge tube. The mixture was shaken using the Touch-Mixer for 1 min and centrifuged for 10 min at 12000 rpm. The supernatant was removed with an insulin syringe and filtered through a Millipore filter (Type H, pore size 0.45 1 μm). The solution was analyzed without any further dilution by HPLC injecting 20 μl of sample. Quantitation was done by a calibration curve.

2.5. In vivo distribution/metabolism studies

Adult, male Sprague-Dawley rats weighing 175–200 g were used. Animals were kept in individual cages with free access to food and water. Groups of at least five rats were used. The investigated compounds were dissolved in DMSO, and the solutions were administered in the tail vein of conscious animals at a dose of 11.3 mg/kg. In the control group (three rats) only the solvent was administered. Animals were sacrificed by decapitation at appropriate time intervals (2, 5, 10, 20, 30, 60 min) after the intravenous injection. Trunk blood was collected into heparinized tubes. The brain, the liver, and the lung were removed and immediately frozen. Samples for HPLC analysis were prepared by homogenizing the organs with isotonic phosphate buffer (pH 7.4). The final concentration of the suspension was 20% (w/w). 400 μl of each of these suspensions were prepared as described previously for HPLC determination.

3. Result and discussion

3.1. Synthesis

The synthetic route for the synthesis of the testosterone anionic chemical delivery system T-aCDS (methyl-pivaloyloxy-methyl-17-testosteryl phosphonate), which includes a biologically active compound and a lipophilic, (acyloxy)alkyl phosphonate type targetor moiety, is summarized in Fig. 2. The methyl-17-testosterylphosphonate (TP^-, 2) was prepared by reaction of testosterone (T,1) with methylphosphonic dichloride in dry pyridine. The reaction was very sensitive to traces of water. The compound was then converted with silver nitrate to give methyl-17-testosterylphosphonate silver salt (3). The silver salt could not be obtained if in the neutralization step some excess of nitric acid was used and/or if the reaction was not carried out in dark. The T-aCDS (6) was prepared by reaction of silver salt (3) with iodomethyl pivalate (5); the latter was formed from chloromethyl pivalate with sodium iodide in dry acetone (dried with anhydrous potassium carbonate and then distilled). During conversion of silver salt to the ester, it is very important to strictly enforce nitrogen atmosphere, dry solvent, and darkness. The T-aCDS obtained was a slightly yellow viscous oil found to be stable in this state at 4°C for more than 1 year if protected from light and moisture.

3.2. In vitro stability studies

The in vitro stability of T-aCDS (6) was examined in various biological matrices at 37°C. The

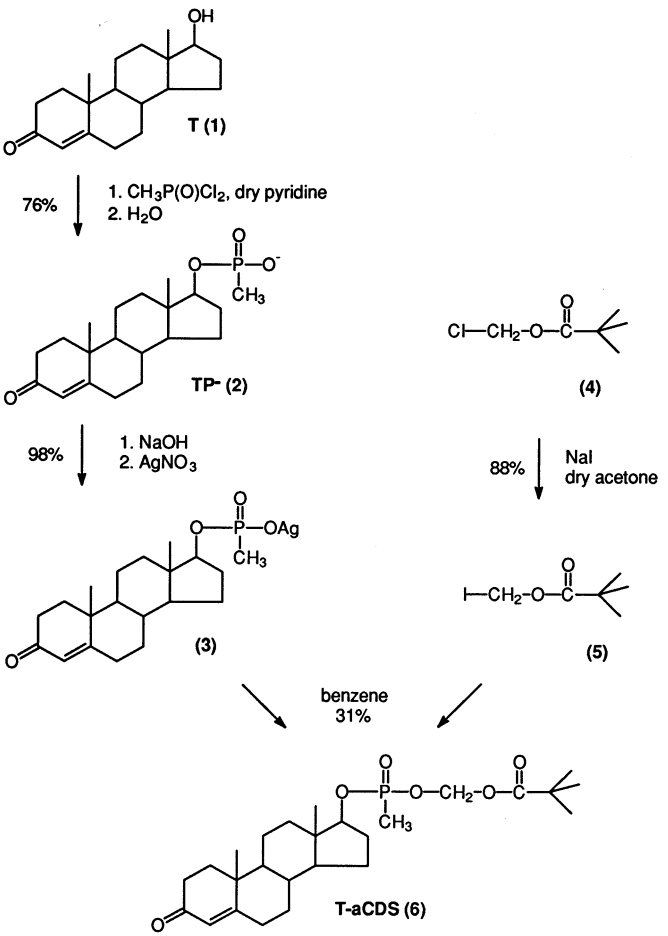

Fig. 2. Synthesis of the anionic chemical delivery system for testosterone (T-aCDS, 6).

aCDS is most stable in brain homogenate but hydrolyses rapidly in liver homogenate. The half-lives ($t_{1/2}$) in different organs are as follows: blood 4.48 min ($r = 0.9388$), lung 5.53 min ($r = 0.9661$), liver 2.82 min ($r = 0.9498$), and brain 7.37 min ($r = 0.9972$) (Fig. 3). When degradation of T-aCDS (6) was monitored in various organ homogenates, its disappearance was observed (Fig. 4a) with the concomitant appearance of TP^- (2) (Fig. 4b). These profiles are consistent with the predicted metabolism of this system.

3.3. In vivo studies

Male SD rats (five per group) were given T-aCDS (6) at a dose of 11.3 mg/kg in DMSO vehicle. Fig. 5a shows the concentration of T-aCDS after i.v. administration. Blood levels were initially the highest but disappeared rapidly with a

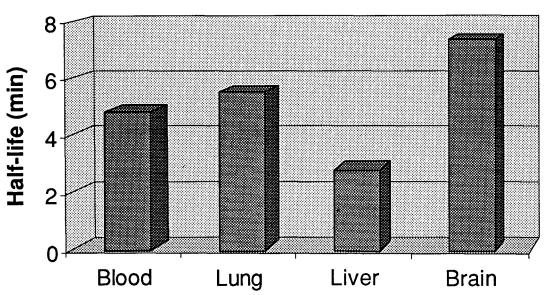

Fig. 3. In vitro half-lives of T-aCDS (6) in different organs. Each value is the mean of seven independent determinations.

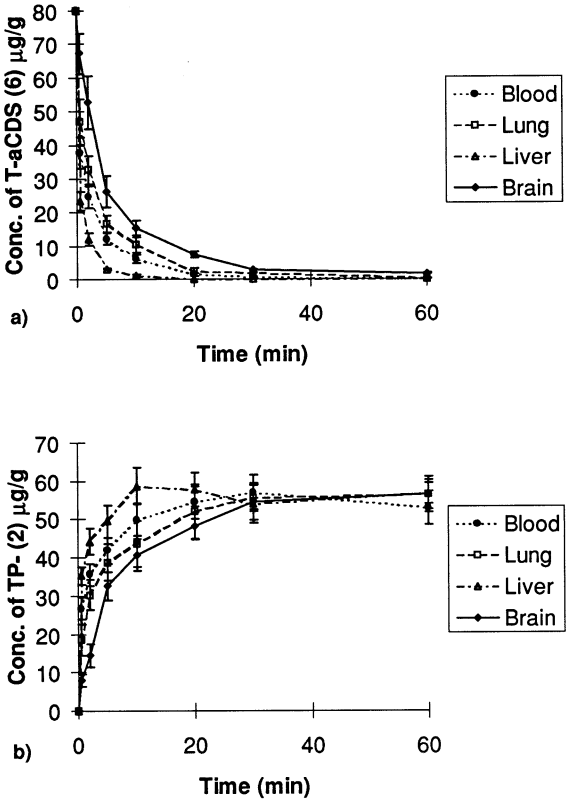

Fig. 4. In vitro concentration change for (a) T-aCDS (6) and (b) the negatively charged intermediate TP^- (2) in various organs.

$t_{1/2}$ of approximately 4 min, and by 30 min no T-aCDS was detectable. For the brain, maximum T-aCDS levels were found 5–10 min after administration; they fell under the borderline of detectability (< 0.1 μg/g) after 60 min. In the lung, the maximum concentration of T-aCDS was higher than in the brain; this value was reached later, after about 10 min, and lung concentration decreased with time after this. The situation was dramatically different for the liver. Due to rapid metabolism, T-aCDS could not be found in detectable concentration during the investigated time intervals.

The concentration change of TP^- (2) after i.v. administration of T-aCDS is shown in Fig. 5b. In liver, TP^- levels were practically at their maximum at the first sampling (5 min); they did not change significantly between 5 and 20 min, and then rapidly disappeared after about 60–65 min. In blood and in lung, highest levels were measured at 20 and 10 min, respectively. Compared to blood levels, maximum values were about 20% lower in the lung and more than 50% lower in the liver.

In the brain, maximum concentration of the decomposition product (TP^-) was obtained at 30 min after administration, indicating that hydrolytic cleavage is slowest here, and this concentration did not decrease significantly during the study, proving that this negatively charged intermediate is indeed 'locked in' the brain. In addi-

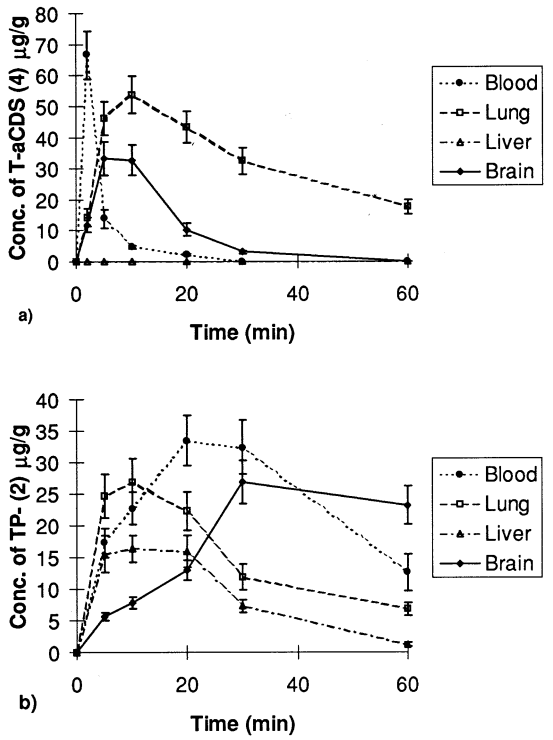

Fig. 5. In vivo concentration change of (a) T-aCDS (6) and (b) the main metabolic product (TP-) (2) in different organs after i.v. administration of a 11.3 mg/kg T-aCDS dose in rat. Each value is the mean of five independent determinations. Due to rapid metabolism, the starting material could not be detected in liver. At the end of observation (60 min) the highest levels of the negatively charged intermediate were detected in the brain (about 25 μg/g). This demonstrates that delivery to the brain and lock-in of the anionic intermediate takes place according to the designed sequence. As this intermediate was fairly resistant to phosphorolytic attack, released testosterone could not be detected.

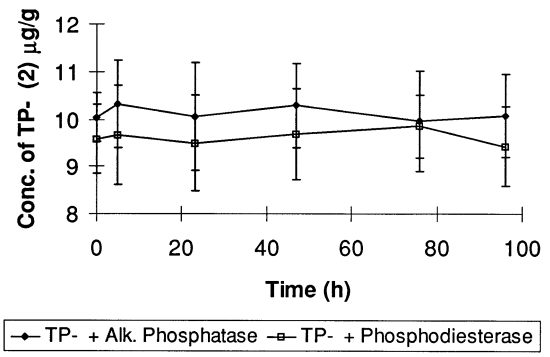

Fig. 6. In vitro enzymatic assay of TP^- with alkaline phosphatase and phosphodiesterase. Released testosterone could not be detected.

tion, when TP^-, and not T-aCDS, was administered i.v., its appearance in the brain could not be detected, an observation that was expected based on the hydrophilic nature of TP^-. These prove that the first step of the designed metabolism works; passive transport through the BBB is followed by hydrolytic cleavage and spontaneous decomposition to provide a locked-in, hydrophylic drug-phosphonate complex.

Unfortunately, during the decrease of the TP^- concentration, no testosterone was detectable. This indicates that this product (TP^-) is resistant to phosphorolytic attack, the second metabolic step. To verify this assumption, in vitro enzymatic assays were performed, and they proved that, indeed, the phosphonate derivative of the secondary hydroxyl group in TP^- is fairly resistant to phosphorolytic attack by alkaline phosphatase or phosphodiesterase (Fig. 6). Similar indications were found in the literature. Phosphodiesterase failed to hydrolyze simple alkyl-monoesters but, interestingly, not aryl-monoesters of phosphoric acids (Landt et al., 1980). Even an ester of a secondary alcohol such as cyclohexanol was not a substrate, although the enzyme readily cleaves the alkyl secondary alcohol ester linkage to the 3′-hydroxyl group of ribose in oligonucleotides, its natural substrates. Phosphodiesterase I and II was found not to hydrolyze an α,β-methylene 5′-phosphonate derivative of AzddTDP within 24 h (Balzarini et al., 1988). An enzymatic degradation study of bis(POM)FdUMP (Farquhar et al., 1994) indicated that this compound is not a substrate for nucleotide metabolizing enzymes as incubation by 10-fold unit excess of alkaline phosphatase, 5′-nucleotidase, or phosphodiesterase I (37°C, 2 h, 0.1 M Tris buffer, pH 8.0) resulted in a degradation rate that was the same as that in buffer alone. Phosphorylation, a well-known reaction (Tasurova et al., 1989) that works in the 'other' direction, is, however, probably a favored in vivo transformation of TP^-.

Design and evaluation of other such anionic chemical delivery systems is under way. As T-aCDS had a hindered phosphonate ester linkage at a secondary alcohol position, another aCDS with less hindered ester functions that should provide easier breakdown was tested. Indeed, here the whole designed metabolic sequence, including release of the active agent, was successfully completed as reported in the second part of this series.

4. Conclusion

A novel, anionic chemical delivery system (aCDS) that uses an (acyloxy)alkyl phosphonate-type targetor moiety for brain-targeted delivery has been designed. For testosterone a (pivaloyloxy)methyl (POM) phosphonate ester derivative has been successfully synthesized to serve as its aCDS. In vitro and in vivo studies confirmed that the first step of the designed metabolism worked; passive transport through the BBB is followed by hydrolytic cleavage and spontaneous decomposition to provide a locked-in, anionic drug-phosphonate complex. However, as the phosphonate derivatives of the secondary hydroxyl group was resistant to alkaline phosphatase or phosphodiesterase, the second step, dephosphorylation, did not take place. Even if release of the active drug could not be detected for this aCDS, the design principle can work for other compounds. A case where the whole designed metabolic sequence is successfully completed and the active agent is released at the targeted site is reported as the second part of this series.

References

Annaert, P., Kinget, R., Naesens, L., De Clercq, E., Augustijns, P., 1997. Transport, uptake and metabolism of the bis(pivaloyloxymethyl)-ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine in an in vitro cell culture system of the intestinal mucosa (Caco-2). Pharm. Res. 14, 492–496.

Balzarini, J., Herdewijn, P., Pauwels, R., Broder, S., De-Clercq, E., 1988. α,β and β,γ-methylene 5′-phosphonate derivatives of 3′-azido-2′, 3′-dideoxythymidine-5′-triphosphate. Biochem. Pharm. 37, 2395–2403.

Bodor, N., 1987. Redox drug delivery systems for targeting drugs to the brain. Ann. NY Acad. Sci. 507, 289–306.

Bodor, N., 1992. New methods of drug targeting. In: Sarel, S., Mechoulam, R., Agranat, l. (Eds.), Trends in Medicinal Chemistry '90, Blackwell, Oxford, pp. 35–44.

Bodor, N., 1995a. Targeted drug delivery via phosphonate derivatives. US Patent 5413996, May 9.

Bodor, N., 1995b. Targeting drugs to the brain by sequential metabolism. In: Rapaka, R., Sorer, H. (Eds.), NIDA Research Monograph Series, Vol. 147, Discovery of Novel Opioid Medications, NIDA, Rockville, MD, pp. 1–32.

Bundgaard, H., 1985. Design of prodrugs: bioreversible derivatives for various functional groups and chemical entities. In: Bundgaard, H. (Ed.), Design of Prodrugs, Elsevier, Amsterdam, pp. 1–92.

Bodor, N., Brewster, M.E., 1983. Problems of delivery of drugs to the brain. Pharmacol. Ther. 19, 337–386.

Bodor, N., Brewster, M.E., 1991. Chemical delivery systems. In: Juliano, R.L. (Ed.), Targeted Drug Delivery. Vol. 100, Handbook of Experimental Pharmacology, Springer-Verlag, Berlin, pp. 231–284.

Bodor, N., Buchwald, P., 1997. Drug targeting via retrometabolic approaches. Pharmacol. Ther. 76, 1–27.

Bodor, N., Farag, H.H., 1984. Improved delivery through biological membranes XIV: brain-specific, sustained delivery of testosterone using a redox chemical delivery system. J. Pharm. Sci. 73, 385–389.

De Clercq, E., 1991. Broad-spectrum anti-DNA virus and antiretrovirus activity of phosphonylmethoxyalkylpurines and -pyrimidines. Biochem. Pharmacol. 42, 963–972.

De Clercq, E., Holy, A., Rosenberg, I., Sakuma, T., Balzarini, J., Maudgal, P.C., 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature 323, 464–467.

Farquhar, D., Khan, S., Srivastva, D.N., Saunders, P.P., 1994. Synthesis and antitumor evaluation of bis[(pivaloyloxy)methyl] 2′-deoxy-5-fluorouridine 5′-monophosphate (FdUMP): a strategy to introduce nucleoside into cells. J. Med. Chem. 37, 3902–3903.

Farquhar, D., Srivastva, D.N., Kuttesch, N.J., Saunders, P.P., 1983. Biologically reversible phosphate-protective groups. J. Pharm. Sci. 72, 324–325.

Haugen, H.F., Skrede, S., 1977. Nucleotide pyrophosphatase and phosphodiesterase. 1. Organ distribution and activities in body fluids. Clin. Chem. 23, 1531–1537.

Iyer, R.P., Phillips, L.R., Biddle, J.A., Thakker, D.R., Egan, W., 1989. Synthesis of acyloxyalkyl acylphosphonates as potential prodrugs of the antiviral, trisodium phosphonoformate (Foscarnet sodium). Tetrahedron Lett. 30, 7141–7144.

Jansen, A.B.A., Russell, T.J., 1965. Some novel penicillin derivatives. J. Chem. Soc., 2127–2132.

Krapcho, J., Turk, C., Cushman, D.W., Powell, J. R., DeForrest, J.M., Spitzmiller, E. R., Karanewsky, D.S., Duggan, M., Rovoyak, G., Schwarz, J., Natarajan, S., Godfrey, J.D., Ryono, D.E., Neubeck, R., Atwal, K.S., Petrillo, E.W. Jr., 1988. Angiotensin-converting enzyme inhibitors. Mercaptan, carboxyalkyl dipeptide, and phosphinic acid inhibitors incorporating 4-substituted prolines. J. Med. Chem. 31, 1148–1160.

Krisch, K.. 1971. Carboxylic ester hydrolases. In: Boyer, P.D. (Ed.) The Enzymes, 5. Academic Press, New York, 1971, pp.43–69.

Landt, M., Everard, R.A., Butler, L.G., 1980. 5′-Nucleotide phosphodiesterase: features of the substrate binding site as deduced from specificity and kinetics of some novel substrates. Biochemistry 19, 138–143.

Methoden der Organischen Chemie (Houben-Weyl), 1964. Band Xll/2., Organische Phosphorverbindungen, Teil 2., 4. Ausgabe, Müller, E. (Ed.), George Thieme Verlag, Stuttgart, 1964, pp. 302-306.

Mullersman, G., Derendorf, H., Brewster, M.E., Estes, K.S., Bodor, N., 1988. High performance liquid chromatographic assay of a central nervous system (CNS)-directed estradiol chemical delivery system and its application after intravenous administration in rats. Pharm. Res. 5, 172–177.

Riess, J., Ourisson, G., 1965. Phosphorus derivatives of steroid hormones. III. Steroid methylphosphonates. Bull. Soc. Chim. France. 4, 933–941.

Saperstein, R., Vicario, P.P., Strout, H.V., Brady, E., Slater, E.E., Greenlee, W.J., Ondeyka, D.L., Patchett, A.A., Hangauer, D.G., 1989. Design of a selective insulin receptor tyrosine kinase inhibitor and its effect on glucose uptake and metabolism in intact cells. Biochemistry 28, 5694–5701.

Sarkar, D.K., Friedman, S.J., Yen, S.S.C., Frautschy, S.A., 1989. Chronic inhibition of hypothalamic-pituitary-ovarian axis and body weight gain by brain-directed delivery of estradiol-17β in female rats. Neuroendocrinology 50, 204–210.

Sastry, J.K., Nehete, P.N., Khan, S., Nowak, B.J., Plunkett, W., Arlinghaus, R.B., Farquhar, D., 1992. Membrane-permeable 5′-dideoxyuridine monophosphate analogue inhibits human immunodeficiency virus infection. Mol. Pharmacol. 41, 441–445.

Schultz, C., Vajanaphanich, M., Harootunian, A.T., Sammak, P.J., Barrett, K.E., Tsien, R.Y., 1993. Acetoxymethyl esters of phosphates: enhancement of the permeability and potency of cAMP. J. Biol. Chem. 268, 6316–6322.

Srinivas, R.V., Robbins, B.L., Connelly, M.C., Gong, YF., Bischofberger, N., Fridland, A., 1993. Metabolism and in vitro antiretroviral activities of bis(pivaloyloxymethyl) prodrugs of acyclic nucleoside phosphonates. Antimicrob. Agents Chemother. 37, 2247–2250.

Srivastva, D., Farquhar, D., 1984. Bioreversible phosphate protective groups: Synthesis and stability of model acyloxymethyl phosphates. Bioorg. Chem. 12, 118–129.

Starrett, J.E., Tortolani, D.R., Russel, J., Hitchcock, M.J.M., Whiterock, V., Martin, J.C., Mansuri, M.M., 1994. Synthesis, oral bioavailability determination, and in vitro evaluation of prodrugs of the antiviral agent 9-(2-(phosphonomethoxy)ethyl)adenine (PMEA). J. Med. Chem. 37, 1857–1864.

Starrett, J.E., Tortolani, D.R., Hitchcock, M.J.M., Martin, J.C., Mansuri, M.M., 1992. Synthesis and in vitro evaluation of a phosphonate prodrug: bis(pivaloyloxymethyl) 9-(2-phosphonylmethoxyethyl)adenine. Antiviral Res. 19, 267–273.

Tasurova, N.B., Khorlin, A.A., Kraevskii, A.A., Korneeva, M.N., Nosik, D.N., Kruglov, I.V., Galegov, G.A., Bibilashvili, R.Sh., 1989. Inhibition of HIV reproduction in cell culture by 5′-phosphonates of 3′-azido-2′,3′-dideoxynucleosides. Mol. Biol. Mosk. 23, 1716–1724.