ELSEVIER

International Journal of Pharmaceutics 104 (1994) 157–167

international journal of pharmaceutics

Drug delivery via active transport at the blood-brain barrier: affinity of a prodrug of phosphonoformate for the large amino acid transporter

Ian Walker *, Dave Nicholls, William J. Irwin, Sally Freeman

Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, UK

(Received 7 July 1993; Accepted 21 September 1993)

Abstract

Due to its hydrophilic nature, the antiviral agent phosphonoformate (PFA) is excluded from the CNS by the blood-brain barrier (BBB). Lipophilic triesters of PFA, designed to penetrate the BBB have been found to be unsuitable as prodrugs due to their rapid and complicated hydrolysis. Hydrophilic drugs, such as L-dopa, are known to cross the blood-brain barrier by means of an active amino acid transporter. Thus, the possibility that a PFA-amino acid conjugate may be actively transported at the BBB was investigated. A PFA-L-tyrosine conjugate [sodium 4-(2′-carboxyl-2′-aminoethyl)phenyl methoxycarbonylphosphonate] was synthesised and characterised. Active amino acid transport was studied in vitro using monolayers of porcine brain microvessel endothelial cells. Confluent monolayers were obtained after 4–5 days in culture, and alkaline phosphatase activity and the presence of Factor VIII antigen were demonstrated histochemically. The transport of L-[^{3}H]tyrosine was shown to be temperature- and concentration-dependent and transport constants were calculated to be $K_m = 0.149$ mM and $V_{max} = 3.07$ nmol/h per insert by non-linear regression. L-Dopa and other large amino acids were found to inhibit the transport of L-[^{3}H]tyrosine, consistent with their transport by the amino acid transporter in vivo. The PFA-L-tyrosine conjugate, which was stable under the experimental conditions, also inhibited L-[^{3}H]tyrosine transport indicating that it may be a substrate for active transport at the BBB.

Key words: Active transport; Amino acid transport; Blood-brain barrier; Brain delivery; Cell culture; Phosphonoformate; Prodrug

1. Introduction

The brain microvessel endothelium provides a barrier to the passive transport of hydrophilic drugs into the brain (for reviews, see Oldendorf, 1977; Brightman, 1989). Phosphonoformate (Foscarnet, PFA) is a useful antiviral agent, showing activity against most retroviruses, including HIV (Oberg, 1989). However, PFA is precluded from entering the brain presumably because it is tri-anionic at physiological pH (pK_a values 0.49, 3.41 and 7.27) (Warren and Williams, 1971).

* Corresponding author.

SSDI 0378-5173(93)E0285-X

Lipophilic triesters of phosphonoformate, which were designed to improve transport properties, are unsuitable prodrugs due to their rapid and complicated hydrolysis, involving competitive P–O and P–C bond cleavage reactions (Krol et al., 1991; Mitchell et al., 1991, 1992; Krol and Thatcher, 1993). In contrast, diesters of phosphonoformate are more stable towards hydrolysis, but are considered to be too polar for passive diffusion into the CNS.

The amino acid drugs, L-dopa (Nutt et al., 1984) and methyl dopa (Markovitz and Fernstrom, 1977) are known to be actively transported across the blood-brain barrier. Cytotoxic drugs have been designed with amino acid moieties in an effort to facilitate active transport at the BBB. Melphalan (phenylalanine mustard) has been shown to have some affinity for the large neutral amino acid transporter at the BBB, although insufficient to result in therapeutic drug concentrations (Greig et al., 1987; Takada et al., 1991). More recently DL-NAM, a derivative of melphalan, has been shown to have a very high affinity for the transporter and appears to be a promising CNS-directed agent (Takada et al., 1991, 1992). Four carrier-mediated systems for amino acid transport across the BBB have been characterised in vivo (Pardridge, 1988). Large neutral amino acids (LNAA) have been shown to be transported by the sodium-independent L-(leucine-preferring) system while small neutral amino acids are transported by the sodium-dependent A-(alanine-preferring) system (Oldendorf, 1971; Betz and Goldstein, 1980). The L-system is present in the luminal and abluminal membranes of brain microvessel endothelial cells (BMEC) and is bidirectional, while the A-system is present only in the abluminal membrane and is unidirectional from brain to blood (Betz and Goldstein, 1980). A separate transport system for basic amino acids at the BBB is documented as is a very low capacity transporter for the efflux of acidic amino acids from the brain (Pardridge, 1988). The L-system has been extensively characterised and is of particular interest in the transport of amino acid drugs into the brain. The saturable nature and stereospecificity of the L-system has been demonstrated (Oldendorf, 1971; Betz and Goldstein, 1980). Large hydrophobic amino acids show the highest affinities for the carrier. Some small amino acids, for example, L-alanine, have a lower affinity while others such as glycine do not appear to be transported by the carrier. The basic amino acid L-histidine has an affinity for the carrier comparable to other large amino acids while the other basic and acidic amino acids are not transported.

Bovine brain microvessel endothelial cell (BMEC) monolayers, grown in cell culture on permeable supports, have been used to study both passive and active transport at the BBB (Audus and Borchardt, 1986). The L-system has been studied in vitro using isolated brain capillaries (Betz and Goldstein, 1978). L-Leucine showed cross-inhibition with L-phenylalanine, L-tyrosine, L-methionine, L-histidine, and to a lesser extent with L-alanine. Little or no cross-inhibition was observed with glycine or L-proline. This is in agreement with the inhibition profile observed in vivo. Using cultured BMEC, the A- and L-systems have been characterised in terms of sodium dependence and independence, respectively (Cancilla and DeBault, 1983). Audus and Borchardt (1986) have established an inhibition profile and measured a K_m of 0.18 mM for L-leucine transport across BMEC monolayers in line with observations in vivo.

The present study involves the cell culture of porcine BMEC, grown on 25 mm Falcon Cyclopore inserts. The active transport of L-[^{3}H]tyrosine across porcine BMEC monolayers is characterised, as is the inhibition of its transport by other amino acids and a novel PFA-L-tyrosine conjugate.

2. Materials and methods

High-field NMR spectra, ^{1}H (300 or 250 MHz) and ^{31}P (121.5 or 101 MHz) were recorded on Bruker AC spectrometers. ^{1}H-NMR spectra were referenced to tetramethylsilane and ^{31}P-NMR spectra were referenced to 85% H_3PO_4; positive chemical shifts are downfield from the reference. ^{31}P-NMR are ^{1}H decoupled or ^{1}H coupled as

stated. Mass spectra were recorded by the SERC mass spectrometry service at Swansea University on a VG 7070E instrument under the positive ion FAB (nitrobenzyl alcohol matrix) technique. Infrared spectra were recorded on a Perkin-Elmer 1310 infrared spectrometer. Melting points were measured on an Gallenkamp electrothermal melting point apparatus and are not corrected. TLC was performed using Kieselgel 60 silica gel plates containing a fluorescent indicator. Spots were visualised under 254 nm UV light or with the aid of iodine. Flash chromatography (Still et al., 1978) was performed using Sorbsil C60 silica gel. Elemental analyses were recorded by Butterworth Laboratories Ltd of Teddington, Middlesex. Dichloromethane, acetonitrile and methanol were dried by refluxing with calcium hydride followed by distillation. Chemicals were obtained from Aldrich or Sigma Chemical Co. Methoxycarbonylphosphonic dichloride was prepared by the method of Morita et al. (1980).

2.1. Synthesis of phosphonoformate-L-tyrosine conjugate (4)

L-Tyrosine benzyl ester (1): This was prepared by a similar method to that of Erlanger and Hall (1954) for the preparation of L-tyrosine benzyl ester hydrochloride. L-Tyrosine (2.0 g, 11 mmol) was added to a stirred solution of polyphosphoric acid (5 g) and benzyl alcohol (25 ml) at 90–95°C. After 4 h at 90–95°C the reaction mixture was added to 2 M hydrochloric acid (200 ml) and then washed with ether (100 ml). The ether layer was washed with 2 M hydrochloric acid (2 × 50 ml). All aqueous layers were combined and the pH adjusted to 10 using sodium carbonate. The product was then extracted into ether (3 × 100 ml). The combined ether extracts were dried and evaporated in vacuo to leave a yellow oil. Recrystallisation from ethyl acetate-hexane gave (1) as colourless crystals: 60% (1.8 g, 6.6 mmol); m.p. 119–120°C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.40–7.20 (5H, m), 6.92 (2H, d, $J_{HH} = 8$ Hz), 6.62 (2H, d, $J_{HH} = 8$ Hz), 5.16 (1H, d, $J_{gem} = 12$ Hz, OCH_AH_BPh), 5.11 (1H, d, $J_{gem} = 12$ Hz, OCH_AH_BPh), 3.73 (1H, d d t, $J_{HH} = 6$ Hz, $J_{HH} = 6$ Hz, $J_{HNH} = 8$ Hz, $ArCH_2CHNH_2$), 3.05–2.45 (4H, bs, NH_2 and H_2O), 3.00 (1H, d d, $J_{gem} = 13.5$ Hz, $J_{HH} = 6$ Hz, $ArCH_AH_BC$), 2.81 (1H, d d, $J_{gem} = 13.5$ Hz, $J_{HH} = 6$ Hz, $ArCH_AH_BC$); IR (Nujol mull) 1720 cm^{-1} (C = O), 1600 (C = C).

N-Carbonyloxybenzyl L-tyrosine benzyl ester (2): A solution of benzyl chloroformate (0.60 g, 3.5 mmol) in acetonitrile (10 ml) was added dropwise to a stirred solution of tyrosine benzyl ester (1.0 g, 3.7 mmol) and triethylamine (0.36 g, 3.5 mmol) in dry acetonitrile (40 ml) at 0°C, over 30 min. After 2 h, compound (2) was purified using flash chromatography (ethyl acetate-hexane 2:1) (Still et al., 1978) and recrystallised from ethyl acetate-hexane to give colourless crystals: 42% (0.45 g, 1.5 mmol); m.p. 117–118°C; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.45–7.25 (10H, m), 6.87 (2H, d, $J_{HH} = 8$ Hz), 6.67 (2H, d, $J_{HH} = 8$ Hz), 5.50 (1H, bs, OH), 5.30 (1H, d, $J_{HH} = 8$ Hz, NH), 5.22 [1H, d, $J_{gem} = 12$ Hz, $CC(O)OCH_AH_BPh$], 5.15 [1H, d, $J_{gem} = 12$ Hz, $CC(O)OCH_AH_BPh$, half of this doublet is under the singlet at 5.13], 5.13 [2H, s, $HNC(O)OCH_2Ph$], 4.69 (1H, d d d, $J_{HNH} = 8$ Hz, $J_{HH} = 6$ Hz, $J_{HH} = 6$ Hz, appears as quartet, $ArCH_2CHNH$), 3.09 (1H, d d, $J_{gem} = 13.5$ Hz, $J_{HH} = 6$ Hz, $ArCH_AH_BC$), 3.03 (1H, d d, $J_{gem} = 13.5$ Hz, $J_{HH} = 6$ Hz, $ArCH_AH_BC$); IR (Nujol mull) 1720 cm^{-1} (C = O, ester), 1680 (C = O, carbamate), 1600 and 1500 (C = C); (Found: C, 70.81; H, 5.79; N, 3.60%; $C_{24}H_{23}O_5N$ requires C, 71.11; H, 5.68; N, 3.46%).

Sodium 4-[2′-(benzyloxycarbonyl)-2′-(*N*-carbonyloxybenzylcarbamate)ethyl]phenyl methoxycarbonylphosphonate (3): A solution of methoxycarbonylphosphonic dichloride (Morita et al., 1980) (0.44 g, 2.5 mmol) in dichloromethane (5 ml) was added dropwise to a stirred solution of *N*-carbonyloxybenzyl L-tyrosine benzyl ester (1 g, 2.5 mmol) in dichloromethane (25 ml). After 4 h, the solvent was removed. The residue was dissolved in acetone (10 ml) and added to Amberlite IRC 50 resin (sodium form, 10 g) in water (50 ml). After stirring for 1 h, the resin was removed by filtration. The solvent was removed and the residue was purified by preparative thin-layer chromatography eluting with methanol-dichloromethane (3:17). A band of R_f 0.25–0.45 was

excised from the plate and the silica extracted with methanol (50 ml). The solvent was removed, the residue dissolved in water (30 ml) and freeze drying gave the title compound as a colourless solid: 40% (0.55 g, 1.0 mmol); m.p. 70–75°C; ^{1}H-NMR (250 MHz, CD_3OD) δ 7.40–7.10 (10H, m), 7.08 (2H, d, J_{HH} = 10 Hz), 7.04 (2H, d, J_{HH} = 10 Hz), 5.10 (2H, s, CC(O)OCH_2Ph), 4.96 (2H, s, NC(O)OCH_2Ph), 4.43 (1H, ddd, ArCH_2CHNH, J_{HNH} = 6 Hz, J_{HH} = 9 Hz, J_{HH} = 6 Hz, appears as quartet), 3.68 (3H, s, OCH_3), 3.07 (1H, dd, J_{gem} = 14 Hz, J_{HH} = 6 Hz, ArCH_AH_BC), 2.89 (1H, dd, J_{gem} = 14 Hz, J_{HH} = 9 Hz, ArCH_AH_BC); ^{31}P-NMR (101 MHz, ^{1}H coupled or ^{1}H decoupled, CD_3OD) δ −6.96 (s); IR (Nujol mull) 1730 cm^{-1} (C = O, benzyl ester), 1710 (C = O, methyl ester), 1690 (C = O, carbamate), 1600 and 1510 (C = C); (Found C, 53.01; H, 4.59; N, 2.36%; $C_{26}H_{25}O_9$NPNa requires C, 56.83; H, 4.55, N, 2.55%; $C_{26}H_{25}O_9$NPNa.2H_2O requires C, 53.33; H, 4.96; N, 2.39%).

Sodium 4-(2′-carboxyl-2′-aminoethyl)phenyl methoxycarbonylphosphonate (4): Palladium 10% on activated carbon (50 mg) was added to a solution of (3) (0.5 g, 0.91 mmol) in dry methanol and the mixture shaken under an atmosphere of hydrogen for 4 h. The catalyst was removed by filtration and the methanol removed by evaporation. The residue was dissolved in water (30 ml) and freeze dried to give the title compound as a colourless solid: 84% (0.25 g, 0.77 mmol); m.p. 190–195°C with decomposition; ^{1}H-NMR (250 MHz, D_2O) δ 7.33 (2H, d, J_{HH} = 8.5), 7.19 (2H, d, J_{HH} = 8.5 Hz), 3.99 (1H, dd, J_{HH} = 5 Hz, J_{HH} = 8 Hz, ArCH_2CH), 3.86 (3H, s, OCH_3), 3.32 (1H, dd, J_{gem} = 14.5 Hz, J_{HH} = 5 Hz, ArCH_AH_B), 3.11 (1H, dd, J_{gem} = 14.5 Hz, J_{HH} = 8.5 Hz, ArCH_AH_B); ^{31}P-NMR (101 MHz, ^{1}H coupled and ^{1}H decoupled, D_2O) δ −7.35 (s); IR (Nujol mull) 1700 cm^{-1} (C = O, ester), 1610 (C = O, carboxyl and C = C), 1500 (C = C); (Found C, 34.83; H, 4.50; N, 3.58%; $C_{11}H_{13}O_7$NPNa requires C, 40.62; H, 4.00; N, 4.31%; $C_{11}H_{13}O_7$NPNa.3H_20 requires C, 34.83; H, 5.01; N, 3.69%); m/z (FAB, nitrobenzyl alcohol matrix) 348 (M + Na^+, 69%), 326 (M + H^+, 100), 255 (64). Observed accurate FAB m/z on (M + H^+) gives 326.0406. $C_{11}H_{14}O_7$NPNa requires 326.0404.

2.2. Isolation and culture of porcine brain microvessel endothelial cells

Porcine brain microvessel endothelial cells (BMEC) were isolated by a method similar to that described by Audus and Borchardt (1986) for the isolation of bovine BMEC. Briefly, seven porcine brains were obtained 30–60 min after death and transported in ice-cold minimum essential medium (MEM, Gibco) buffered with 50 mM Hepes, pH 7.4, and containing 50 μg/ml polymyxin B, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Under sterile conditions, the meninges and surface blood vessels were removed, using forceps, and discarded. The grey matter of the cerebral cortex was scraped away with a sterile razor blade and minced into 1–2 mm cubes. Approx. 250 ml of minced grey matter was added to 2.5 g dispase (Boehringer-Mannheim) in 15 ml MEM and placed in a shaking water bath at 37°C. After 30 min 130 ml of MEM pH 9–10 was added to adjust the pH to 7.4. The incubation was continued for a further 2 h. The cell suspension was then poured into two sterile 250 ml centrifuge tubes and centrifuged at 3200 rpm (approx. 1000 × g) for 10 min at 4°C in a JA-14 rotor, in a Beckman J2-21 centrifuge. The brown, supernatant solution was then discarded leaving semi-solid material behind, which was then mixed thoroughly with 500 ml of 13% dextran solution and divided into four 250 ml sterile centrifuge tubes (the 13% dextran solution consisted of 13% dextran [60–90 kDa, Sigma], MEM, 10 mM Hepes [adjusted to pH 7.4], 50 μg/ml polymyxin B, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B). The tubes were then centrifuged as before, except at 7700 rpm (approx. 6000 × g). The red pellet and surrounding material was retained and the floating matter and dextran solution were discarded. The four red pellets were each resuspended in 5 ml of MEM containing 5 mg collagenase/dispase (Boehringer-Mannheim). These were combined in a sterile 50 ml tube and placed in a shaking water bath at 37°C for 4.5 h. After the incubation, the cell suspension was diluted to 50 ml with MEM and centrifuged at 2800 rpm (approx. 1000 × g) for 10 min in a Sarstedt LC1 centrifuge. The

pellet was resuspended in a total volume of 8 ml with MEM. A 2 ml portion of this suspension was carefully layered on top of four pre-established 50% Percoll (Sigma) gradient tubes and centrifuged at 2800 rpm for 10 min (the pre-established 50% Percoll gradient tubes consisted of 50% Percoll, MEM, 10 mM Hepes [adjusted to pH 7.4], 50 μg/ml polymyxin B, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B, and centrifuged at 20000 × *g* for 60 min at 4°C). The portion of the Percoll containing the endothelial cells was then removed and diluted to 50% with MEM and centrifuged at 2800 rpm for 10 min. The pellet was resuspended in 40 ml of medium containing equal parts of F12 Hams nutrient mix (Gibco) and MEM, 10 mM Hepes (adjusted to pH 7.4), 13 mM sodium bicarbonate, 50 μg/ml polymyxin B, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B supplemented with 10% DMSO and 20% plasma-derived horse serum (HyClone). The cell suspension was then filtered through 200 μm nylon mesh. For later use, 1.5 ml portions of suspension were placed in Cryovials and frozen in a liquid nitrogen cell bank. Cell counts were performed by crystal violet nuclei staining and viability was estimated to be greater than 90% by trypan blue exclusion. Endothelial cells were seeded at a density of 50000 cells/cm^2 onto 25 mm Falcon cell culture inserts (Becton Dickinson) coated with rat-tail collagen and bovine fibronectin (Sigma). The cells were incubated at 37°C in 100% humidity and 5% carbon dioxide in air. The cells were left undisturbed for 3 days, then the medium was changed. Culture medium consisted of equal parts of F12 Hams nutrient mix and MEM, 10 mM Hepes (adjusted to pH 7.4), 13 mM sodium bicarbonate, 100 μg/ml heparin, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B supplemented with 10% plasma-derived horse serum. Medium was changed after a further 2 days in culture. Confluent BMEC monolayers were formed after 4–5 days in culture and used in transport studies on day 7 of culture. Alkaline phosphatase activity (using Sigma kit no. 85) and the presence of Factor VIII antigen (using a Dako primary antibody and Vectastain reagents) were demonstrated on 7-day-old confluent BMEC monolayers, by histochemical methods.

2.3. Transport studies

Three 7-day-old confluent BMEC monolayers in Falcon 25 mm inserts were removed from their six-well plate and the medium removed by aspiration. Transport medium (Hank's Balanced Salt Solution buffered to pH 7.4 with 14 mM Hepes) (1.5 ml) containing 0.2 μCi of [^{14}C]mannitol (60 mCi/mmol, Amersham) and 0.2 μCi of L-[^{3}H]tyrosine (57 Ci/mmol, Amersham) were added to each insert. Each insert was placed in a six-well plate containing transport medium (2.5 ml) in each well. At the end of the incubation period, the inserts were removed and the solution from the inserts were individually added to Optiphase HiSafe 3 scintillation cocktail (LKB, 10 ml). The contents of the wells were also individually added to scintillation cocktail (10 ml). The membrane was removed from the insert with a scalpel and added to scintillation cocktail (10 ml). All samples were then counted using a Packard Tri-Carb 1600TR liquid scintillation counter and the transport of [^{14}C]mannitol and L-[^{3}H]tyrosine determined.

2.4. Temperature dependence

The transport of 0.2 μCi of [^{14}C]mannitol (60 mCi/mmol, Amersham) and 0.2 μCi of L-[^{3}H]tyrosine (57 Ci/mmol, Amersham) through the BMEC monolayers were monitored at 37°C and 4°C. After 30 min, the inserts were moved to other wells containing fresh transport buffer. This was repeated at 30 minute intervals up to a total incubation period of 120 min. The transport medium from the wells in the six-well plates were individually counted and the cumulative transport with time was calculated.

2.5. Concentration dependence

The transport of 0.2 μCi of [^{14}C]mannitol (60 mCi/mmol) and 0.2 μCi of L-[^{3}H]tyrosine (57 Ci/mmol) was monitored at 37°C for 60 min, in the presence of a range of L-tyrosine concentrations (0.005–1 mM) in the apical chamber. Kinetic parameters were calculated using Enzpack

(Biosoft Ltd), Fig P (Biosoft Ltd) and NONREG (Irwin, 1990).

2.6. Inhibition profile

The transport of 0.2 μCi of [^{14}C]mannitol (60 mCi/mmol) and 0.2 μCi of L-[^{3}H]tyrosine (57 Ci/mmol) was monitored at 37°C for 60 min, in the presence of a range of potential inhibitors of L-tyrosine transport (2 mM) in the apical chamber. Any inhibition observed was expressed as a percentage of the transport measured without any potential inhibitor (control).

2.7. Correction of transport data

All L-[^{3}H]tyrosine transport data were corrected for paracellular leakage, using a method analogous to that of Audus and Borchardt (1986). In all experiments the transport of [^{14}C]mannitol and L-[^{3}H]tyrosine was measured simultaneously to give an estimate of paracellular diffusion (PD). The flux of [^{14}C]mannitol was multiplied by a factor (K), to account for the relative diffusion rates of mannitol and L-tyrosine. This was subtracted from the flux of L-[^{3}H]tyrosine, using the following equations:

$$\text{corrected \% transport of L-[}^{3}\text{H]tyrosine} = \text{observed \% transport of L-[}^{3}\text{H]tyrosine} - (\text{PD} \times K) \quad (1)$$

or

$$\text{corrected transport of L-[}^{3}\text{H]tyrosine (cpm)} = \text{observed transport of L-[}^{3}\text{H]tyrosine (cpm)} - [\text{total L-[}^{3}\text{H]tyrosine (cpm)} \times \text{PD} \times 0.01 \times K] \quad (2)$$

where PD denotes the estimate of paracellular diffusion (simultaneous [^{14}C]mannitol % transport) and

K = (diffusion of L-tyrosine across a permeable support) /(diffusion of mannitol across a permeable support)

The value of K was determined experimentally from the relative diffusion rates of L-[^{3}H]tyrosine (19.48%/h) and [^{14}C]mannitol (21.43%/h) across a cyclopore membrane (Falcon 25 mm insert) and was found to be 0.9. This experimental determination of K differs from the theoretical method used by Audus and Borchardt (1986) in which the ratio of the square roots of the molecular weights is used ($K = \sqrt{M_{\text{Man}}}/\sqrt{M_{\text{Tyr}}}$). This method gives a value of K for L-[^{3}H]tyrosine/[^{14}C]mannitol of 1.0, although a significant experimental difference in their rates of diffusion can be demonstrated. This correction showed that the transcellular diffusion of tyrosine was very low, and that the tyrosine flux was essentially due to active transport.

2.8. Identification of L-[^{3}H]tyrosine post-transport using radio-thin-layer chromatography

After a 180 min transport experiment using 1.0 μCi of L-[^{3}H]tyrosine, a sample of medium from the basolateral chamber was applied in spots across the width of a Merck aluminium-backed cellulose TLC plate (3.5 cm wide and 4.5 cm long). The plate was eluted with pyridine-*n*-butanol-water (1:1:1). The solvent was allowed to run 2.5 cm up the plate before it was removed from the solvent tank and dried. The plate was then cut into ten 2.5 mm horizontal strips from the baseline to the solvent front. Each strip was individually added to Optiphase HiSafe 3 scintillation cocktail (10 ml) and counted using a Packard Tri-Carb 1600TR liquid scintillation counter. The distance from the baseline was then plotted against dpm. This was repeated using L-[^{3}H]tyrosine, not subjected to transport, for use as a reference.

2.9. Identification of L-tyrosine-PFA conjugate (4) post-transport

A transport study was performed using 2 mM L-tyrosine-PFA conjugate (4) in the apical transport medium. After 180 min at 37°C, a sample of the basolateral medium (1 ml) was taken and the ^{31}P-NMR spectrum of the sample recorded, using a D_2O inner lock. This was compared with a spectrum of (4) dissolved in transport medium.

3. Results and discussion

The phosphonoformate-L-tyrosine conjugate (4) was synthesised as shown in Fig. 1. The key step was the reaction between methoxycarbonylphosphonic dichloride (Morita et al., 1980) with one equivalent of protected L-tyrosine (2). The remaining chloro function was hydrolysed and converted to the sodium salt using Amberlite IRC 50 anion-exchange resin (sodium form) to give (3). Finally, the protecting groups were removed by catalytic hydrogenation to give (4). All ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were consistent with structures (1)–(4). Compounds (2)–(4) were characterised by elemental analysis and the FAB mass spectrum was recorded on compound (4).

Seeding of 50000 cells/cm^2 resulted in confluent monolayers of spindle-shaped cells after 4–5 days in culture. The monolayers provided the greatest barrier to paracellular diffusion on day 7 of culture as indicated by [^{14}C]mannitol transport (data not shown). [^{14}C]Mannitol transport reached a minimum around day 7 (typically 2–3%/h) followed by a progressive increase in transport with days in culture. Transport studies were therefore performed on day 7. The stability of L-[^{3}H]tyrosine under transport conditions was investigated by radio-TLC, which showed a high degree of coincidence between the histograms for the sample taken from the basolateral chamber, after a 3 h transport experiment at 37°C, and a L-[^{3}H]tyrosine reference. This confirmed that L-[^{3}H]tyrosine was not significantly metabolised during transport across the monolayer and that the ^{3}H label detected in the basolateral chamber was L-[^{3}H]tyrosine as shown in Fig. 2.

Transport of tyrosine across the BMEC monolayers may entail mediation by an active carrier system, passive transcellular and paracellular diffusion. As shown in Eq. 3, the amino acid flux (J), from a substrate solution of concentration [S], is thus dependent upon the Michaelis-Menten

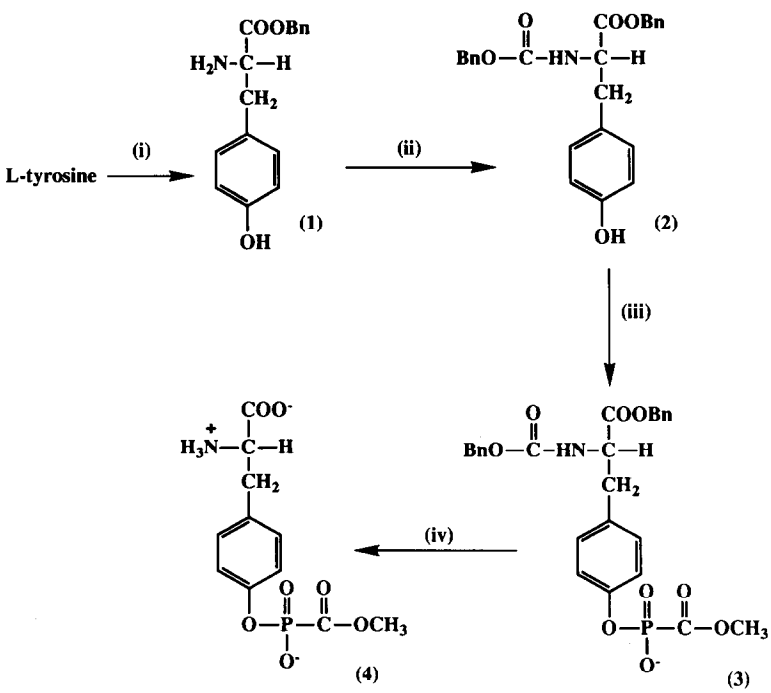

Fig. 1. Synthesis of a phosphonoformate-L-tyrosine conjugate (4): (i) benzyl alcohol/polyphosphoric acid; (ii) benzyl chloroformate; (iii) methoxycarbonylphosphonic dichloride; (iv) hydrogen/palladium catalyst.

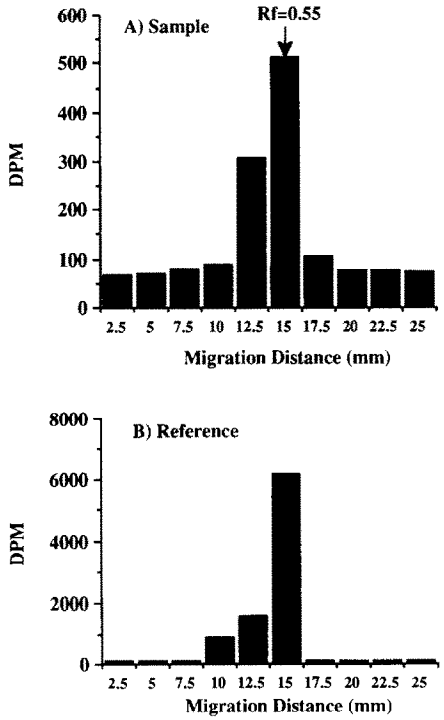

Fig. 2. The identification of L-[^{3}H]tyrosine post-transport using radio-TLC.

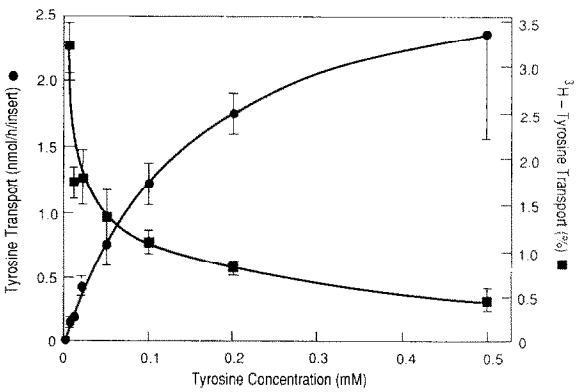

Fig. 3. Concentration dependence of tyrosine transport.

parameters (K_m and V_{max}) for the active component, and the transcellular (k_d) and paracellular (k_p) diffusion rates.

$$J = (V_{max} \cdot [S])/(K_m + [S]) + k_d[S] + k_p[S] \quad (3)$$

The correction with mannitol effectively eliminates the paracellular component of transport due to a leaky monolayer. In order to assess the

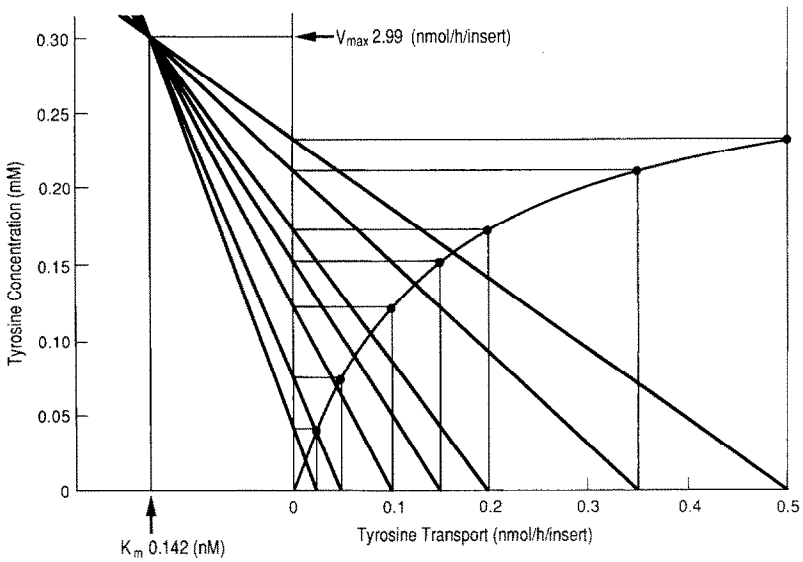

Fig. 4. Direct linear method of calculating kinetic parameters for the transport of L-[^{3}H]tyrosine.

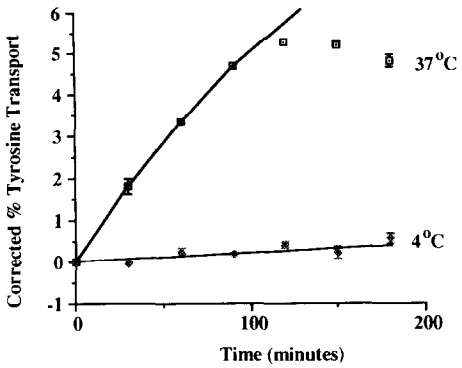

Fig. 5. Temperature dependence of L-[^{3}H]tyrosine transport.

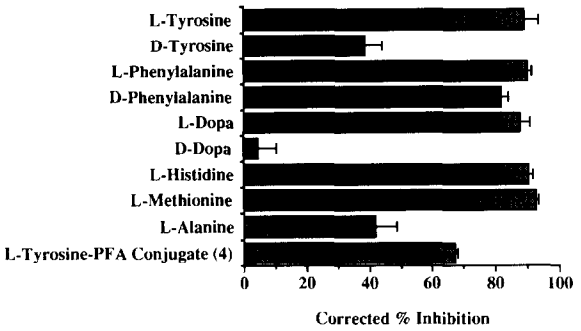

Fig. 6. Cross-inhibition of L-[^{3}H]tyrosine with other amino acids at 2 mM concentration.

importance of the other two pathways, and to identify any saturable component of L-[^{3}H]tyrosine transport, the effect of increasing unlabelled tyrosine concentration upon L-[^{3}H]tyrosine transport was studied. Fig. 3 shows a plot of the corrected data of radiolabelled and total tyrosine flux, and indicates that a maximum plateau value is attained. Non-linear regression of the transport data to Eq. 3 ($k_p = 0$) confirms that transcellular diffusion is inconsequential ($k_d \approx 0$). Thus, tyrosine transport across the BMEC monolayer is saturable; this is characteristic of Michaelis-Menten kinetics (Eq. 3; $k_p = 0$, $k_d = 0$) and indicates the availability of a finite number of transporters at the cell surfaces.

Using a variety of linear transformations (Eadie-Hofstee, Lineweaver-Burk, Hanes-Woolf), the data gave the transport constants in Table 1. Kinetic constants were also calculated from the direct linear method (Enzpack, Biosoft) as shown in Fig. 4, and from non-linear regression (Fig P, Biosoft and NONREG, Irwin, 1990) to Eq. 3. The values obtained by these methods are also recorded in Table 1 and show close agreement. The K_m values were also in agreement with the K_m value for L-tyrosine transport calculated in vivo (0.15 mM; Pardridge, 1983).

A comparison of the transport of L-[^{3}H]tyrosine was made at 4 and 37°C (Fig. 5). It can be seen that, after correcting for the paracellular component, the transport of L-[^{3}H]tyrosine is almost abolished at 4°C which is a feature expected of transport utilising an active carrier system. However, it can also be seen that the tyrosine flux at 37°C is only linear for a period of 90 min. Subsequent experiments were, therefore, limited to a period of 60 min.

To provide a profile of the specificity of the tyrosine transporter, the effect of various amino acids upon the transport of L-[^{3}H]tyrosine was investigated. A range of competitors, at concentrations (2 mM) which far exceeded that of L-tyrosine (2×10^{-6} mM) was studied and the inhibition profile is shown in Fig. 6.

The transport showed stereospecificity with the L-enantiomers showing greater inhibition of L-

Table 1
Michaelis-Menten parameters for the transport of L-[^{3}H]tyrosine across BMEC monolayers

Method	Ordinate	Abscissa	K_m (mM)	V_{max} (nmol/h per insert)	r
Eadie-Hofstee	J	J/[S]	0.135	2.93	0.992
Lineweaver-Burk	$1/J$	1/[S]	0.137	2.98	0.999
Hanes-Woolf	[S]/J	[S]	0.144	3.04	0.999
Direct method	J	[S]	0.142	2.99	–
Non-linear regression	J	[S]	0.149	3.07	0.997

[^{3}H]tyrosine transport than the D-enantiomers. L-Dopa showed complete inhibition (87.8 ± 2.9%) while no inhibition was demonstrated with D-dopa (4.5 ± 6.0%) and L-tyrosine showed complete inhibition (89.2 ± 4.2%) while D-tyrosine showed partial inhibition (38.8 ± 5.4%). This is in general agreement with previous work in vivo (Oldendorf, 1973) and in vitro (Audus and Borchardt, 1986). Stereospecificity could not be demonstrated with phenylalanine as both the L- and D-enantiomer showed complete inhibition (90.2 ± 1.3 and 81.7 ± 2.6%, respectively). This is in contrast with previous studies (Audus and Borchardt, 1986). However, the relative order of stereospecificity observed for dopa, tyrosine and phenylalanine is consistent with the work of Oldendorf (1973), who showed transport of dopa to be highly L-enantiomer-specific while that of tyrosine and phenylalanine was progressively less stereospecific. The large basic amino acid L-histidine and the sulphur-containing L-methionine showed complete inhibition of L-[^{3}H]tyrosine transport (90.6 ± 1.1 and 92.6 ± 1.1%, respectively) whereas the small amino acid L-alanine showed only partial inhibition (42.2 ± 6.7%) in agreement with previous work (Oldendorf, 1971; Betz and Goldstein, 1978).

The L-tyrosine linked phosphonoformate conjugate (4) was shown to inhibit the transport of L-[^{3}H]tyrosine by 67.3 ± 0.6%. The stability of the conjugate (4) was confirmed post-transport which excluded the possibility that the observed inhibition was due to the formation of L-tyrosine from the hydrolysis of (4). The identification of (4) in the basolateral transport medium was confirmed by ^{31}P-NMR spectroscopy which gave a single peak at δ −7.29 ppm. The chemical shift was confirmed with an authentic sample. The inhibition of L-[^{3}H]tyrosine transport is consistent with (4) being a substrate for the amino acid carrier, however the possibility exists that it may be an inhibitor. Further studies to exclude this possibility will require development of a highly sensitive HPLC assay for (4) or by the radiolabelling of (4).

A high affinity for the amino acid carrier would be required if this compound were to achieve therapeutic concentrations in the brain. For example, melphalan has insufficient affinity for the carrier to achieve significant brain uptake (Takada et al., 1991). Modifying melphalan to give DL-NAM has enhanced affinity and achieved a greater brain uptake (Takada et al., 1992). DL-NAM has been reported to have a transport inhibition constant (K_i) approximately two orders of magnitude less than the K_m values of endogenous amino acid (Takada et al., 1991). This indicates the very high affinity of DL-NAM for the amino acid carrier. The K_i for the PFA-tyrosine conjugate (4) was estimated using Eq. 4, derived from the Michaelis-Menten expressions for transport with (J) and without (J_i) the presence of a reversible inhibitor, concentration [I] and inhibition constant K_i, where the percentage inhibition of transport in the presence of the inhibitor is $R_{100} = 100(J - J_i)/J$. At low concentrations of the transported molecule [S], $K_m \gg [S]$ and the expression then approximates to Eq. 5 (Takada et al., 1991).

$$K_i = \frac{K_m \cdot [I]}{\frac{100 \cdot (K_m + [S])}{100 - R_{100}} - K_m - [S]} \quad (4)$$

$$K_i = \frac{(100 - R_{100}) \cdot [I]}{R_{100}} = \frac{100 \cdot [I]}{R_{100}} - [I] \quad (5)$$

The K_i by this procedure was estimated to be 0.86 mM. As this is greater than the K_m of L-tyrosine measured previously (0.15 mM) it is unlikely that the conjugate will have sufficient affinity for the carrier to achieve therapeutic concentrations. Transport of this derivative at the BBB is likely to be limited by competition with other endogenous amino acids. A modification similar to that used for DL-NAM, using a 2-amino-1,2,3,4-tetrahydro-2-naphthoic acid moiety in place of a conventional α-amino acid structure, may increase the affinity of a PFA conjugate for the BBB carrier.

As the conjugate (4) is inactive against HIV, hydrolysis to PFA within the brain is required. Therefore, the rate of hydrolysis of this, or analogous compounds, in plasma would have to be sufficiently slow to allow the prodrug to gain access to the brain but not so slow that it would remain inactive within the brain. The stability of

(4) has been confirmed under transport experiment conditions. Its stability in vivo, in particular in plasma and brain must be elucidated.

4. Acknowledgements

We thank the MRC AIDS Directed Programme for a studentship (I.W.), a travel award (D.N.) and a project grant, and the Lister Institute for a fellowship (S.F.) We would also like to thank Professors R.T. Borchardt and K.L. Audus for allowing D.N. to study cell culture methods at the University of Kansas.

5. References

Audus, K.L. and Borchardt, R.T., Characteristics of the large neutral amino acid transport system of bovine brain microvessel endothelial cell monolayers. *J. Neurochem.*, 47 (1986) 484–488.

Betz, A.L. and Goldstein, G.W., Polarity of the blood-brain barrier: Neutral amino acid transport into isolated brain capillaries, *Science,* 202 (1978) 225–226.

Betz, A.L. and Goldstein, G.W., The basis of active transport at the blood-brain barrier, *Adv. Exp. Med. Biol.*, 131 (1980) 5–16.

Brightman, M.W., The anatomical basis of the blood-brain barrier. In Neuwelt, E.A. (Ed.), *Implications of the Blood-Brain Barrier and its Manipulation,* Plenum, New York, 1989, pp. 53–78.

Cancilla, P.A. and DeBault, L.E., Neutral amino acid transport properties of cerebral endothelial cells in vitro. *J. Neuropathol. Exp. Neurol.*, 42 (1983) 191–199.

Erlanger, B.F. and Hall, R.M., The improved synthesis of amino acid benzyl esters. *J. Am. Chem. Soc.*, 76 (1954) 5781–5782.

Greig, N.H., Momma, S., Sweeney, D.J., Smith, Q.R. and Rapoport, S.I., Facilitated transport of melphalan at the rat blood-brain barrier by the large neutral amino acid carrier system. *Cancer Res.*, 47 (1987) 1571–1576.

Irwin, W.J., *Kinetics of Drug Decomposition: BASIC Computer Solutions,* Elsevier, Amsterdam, 1990, pp. 175–181.

Krol, E.S., Davis, J.M. and Thatcher, G.R.J., Hydrolysis of phosphonoformate esters: Product distribution and reactivity patterns. *J. Chem. Soc. Chem. Commun.*, (1991) 118–119.

Krol, E.S. and Thatcher, G.R.J., Hydrolysis of phosphonoformate triesters: Rate acceleration of a millionfold in nucleophilic substitution at phosphorus. *J. Chem. Soc. Perkin Trans. 2,* (1993) 793–794.

Markovitz, D.C. and Fernstrom, J.D., Diet and uptake of Aldomet by the brain: competition with natural large neutral amino acids. *Science,* 197 (1977) 1014–1015.

Mitchell, A.G., Nicholls, D., Irwin, W.J. and Freeman, S., Prodrugs of phosphonoformate: The effect of *para*-substituents on the products, kinetics and mechanism of hydrolysis of dibenzyl (methoxycarbonyl)phosphonate. *J. Chem. Soc. Perkin Trans. 2,* (1992) 1145–1151.

Mitchell, A.G., Nicholls, D., Walker, I., Irwin, W.J. and Freeman, S., Prodrugs of phosphonoformate: products, kinetics and mechanisms of hydrolysis of dibenzyl (methoxycarbonyl)phosphonate. *J. Chem. Soc. Perkin Trans. 2,* (1991) 1297–1303.

Morita, T., Okamoto, Y. and Sakurai, H., A mild and facile synthesis of alkyl and arylphosphonyl dichlorides under neutral conditions. Reaction of bis(trimethylsilyl) phosphonates with PCl_5. *Chem. Lett.*, (1980) 435–438.

Nutt, J.G., Woodward, W.R., Hammerstad, J.P., Carter, J.H. and Anderson, J.L., The 'on-off' phenomenon in Parkinson's disease – relation to levodopa absorption and transport. *N. Engl. J. Med.*, 310 (1984) 483–488.

Öberg, B., Antiviral effects of phosphonoformate (PFA, foscarnet sodium). *Pharmacol. Ther.*, 40 (1989) 213–285.

Oldendorf, W.H., Brain uptake of radiolabelled amino acids, amines and hexoses after arterial injection. *Am. J. Physiol.*, 221 (1971) 1629–1639.

Oldendorf, W.H., Stereospecificity of blood-brain barrier permeability to amino acids. *Am. J. Physiol.*, 224 (1973) 967–969.

Oldendorf, W.H., The blood-brain barrier. *Exp. Eye Res.*, (Suppl.) (1977) 177–190.

Pardridge, W.M., Brain metabolism: A perspective from the blood-brain barrier. *Physiol. Rev.*, 63 (1983) 1481–1535.

Still, W.C., Kahn, M. and Mitra, A., Rapid chromatographic technique for preparative separations with moderate resolution, *J. Org. Chem.*, 43 (1978) 2923–2925.

Takada, Y., Greig, N.H., Vistica, D.T., Rapoport, S.I. and Smith, Q.R., Affinity of antineoplastic amino acid drugs for the large neutral amino acid transporter of the blood-brain barrier. *Cancer Chemother. Pharmacol.*, 29 (1991) 89–94.

Takada, Y., Vistica, D.T., Greig, N.H., Purdon, D., Rapoport, S.I. and Smith, Q.R., Rapid high affinity transport of a chemotherapeutic amino acid across the blood-brain barrier. *Cancer Res.*, 52 (1992) 2191–2196.

Warren, S. and Williams, M.R., The acid-catalysed decarboxylation of phosphonoformic acid. *J. Chem. Soc. B,* (1971) 618–621.