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Evidence for P-glycoprotein-modulated penetration of morphine-6-glucuronide into brain capillary endothelium

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1 Morphine-6-glucuronide is one of the major metabolites of morphine. The potent analgesic action of this compound together with its potential lower apparent toxicity in man, when compared with morphine, indicated its clinical importance.

2 Primary cultures of porcine brain capillary endothelial cells were used to study brain penetration of morphine-6-glucuronide. Biochemical characterization of the cell cultures revealed a marked enrichment in enzymatic activity of alkaline phosphatase (56 fold) and angiotensin converting enzyme (230 fold) as compared to whole brain tissue. By immunostaining the presence of vimentin, factor VIII, the tight junction associated protein ZO-1, and P-glycoprotein was shown. Functional characterization revealed that the carrier system responsible for transport of neutral amino acids was intact.

3 Uptake and transport of morphine-6-glucuronide was marginal and in the range of the extracellular marker sucrose. However, uptake of morphine-6-glucuronide was enhanced significantly ($P<0.0001$) in presence of the inhibitors of P-glycoprotein, verapamil or vincristine. The finding that morphine-6-glucuronide may serve as a substrate for P-glycoprotein was confirmed in multidrug-resistant P388 tumour cells.

4 We conclude that penetration of the blood-brain barrier by morphine-6-glucuronide may depend on the expression of the product of the multidrug-resistance (MDR) gene in brain capillary endothelial cells.

Keywords: Blood-brain barrier; brain; endothelial cells; transport; morphine; morphine-glucuronides; morphine-6-glucuronide

Introduction

Morphine-6β-glucuronide (M-6-G) significantly contributes to the apparent analgesic efficacy of morphine (Osborne *et al.*, 1992). In disease states such as renal failure or during chronic dosing of morphine, morphine glucuronides may easily accumulate in the blood to levels greater than those of morphine (Osborne *et al.*, 1992). Pharmacological characterization of M-6-G (Paul *et al.*, 1989) revealed that it produced analgesia with an effect approximately twice that of morphine after peripheral administration. When injected directly into the central nervous system (Paul *et al.*, 1989), either intracerebro-ventricularly or intrathecally, M-6-G showed a 90 and 650 fold greater relative potency than morphine in the tail flick latency test in mice. Thus, variations in morphine metabolism may contribute to the marked variation in the effective dose of morphine required for analgesia (McQuay *et al.*, 1990). Like morphine, M-6-G is relatively selective for μ opiate receptors in the brain, binding to these receptors with higher affinity than to κ or δ receptors (Pasternak *et al.*, 1987). There is no significant difference in binding affinity of M-6-G and morphine to the μ_1 receptor but a 4 to 5 fold lower affinity of M-6-G for the μ_2 receptor. These different affinities for μ subtypes might offer an explanation for the lower risk of M-6-G in inducing respiratory depression and gastrointestinal effects in man (Hucks *et al.*, 1992; Thompson *et al.*, 1995).

Despite the fact that glucuronidation of morphine results in an increased hydrophilicity of the metabolites, it has been demonstrated that M-6-G is able to penetrate the brain in rats although the rate of penetration is much slower for M-6-G than for morphine (Yoshimura *et al.*, 1973). At present, however, it remains unclear by which mechanisms M-6-G might cross the blood-brain barrier. Based on force-field and quantum mechanical calculations on M-6-G it was suggested (Carrupt *et al.*, 1991) that M-6-G might have a greater than expected lipophilicity due to self-coiling of the molecule. This may allow morphine-glucuronides to cross the blood-brain barrier by simple diffusion. Indirect evidence from a recent study in the neonatal guinea-pig brain (Murphey & Olsen, 1994) seems to support this view. Other authors (Polt *et al.*, 1994) speculated, that glucose transporter GLUT-1 might be responsible for the transport of β-D-glucosylated peptides, a mechanism which could also apply for the 6-glucuronide metabolite of morphine. From studies on renal elimination of morphine and its glucuronides (Crugten *et al.*, 1991) it was proposed that, at least in the kidney, a carrier-mediated transport system for M-6-G might exist.

This study was undertaken to identify factors modulating the brain uptake of M-6-G. Uptake and transport properties of M-6-G were determined in primary cultures of porcine brain capillary endothelial cells, being the main constituents of the blood-brain barrier. Cultured brain capillary endothelial cells have recently been demonstrated to possess the major functional characteristics of the blood-brain barrier *in vivo* (Pardridge *et al.*, 1990; Dehouck *et al.*, 1992; Chesne *et al.*, 1993).

Methods

Octanol to 100 mM phosphate buffer (pH 7.0) partition coefficients were determined following equilibration over 24 h at room temperature using isotope labelled tracers (Amersham, UK or Du Pont NEN, U.S.A.). [^{3}H]-M-6-G was synthesized according to Yoshimura *et al.* (1973) using 10 mg [^{3}H]-morphine as an educt. Purification was performed by preparative reversed-phase h.p.l.c. (isocratic separation, stationary phase Nucleosil 7 C_8 (Macherey Nagel, Germany), preparative column 250 x 10 mm i.d., mobile (volatile) phase was methanol:isopropanol:H_2O = 5:2.5:92.5 (v/v/v)). Identity and purity of the reaction product was assayed by u.v.-Vis and fluorescence spectroscopy, reversed-phase h.p.l.c. (Huwyler *et al.*, 1995), fast atomic bombardment mass spectroscopy

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(FABS) and nuclear magnetic resonance (^{13}C-n.m.r. and ^{1}H-n.m.r.) (Carrupt *et al.*, 1991). Based on ^{1}H-n.m.r. spectra, the purity of M-6-G was estimated to be greater than 99%. Enzymatic activity of alkaline phosphatase was determined by use of P-nitrophenyl-phosphate as a substrate (Goldstein *et al.*, 1975). Angiotensin converting enzyme was determined as described by Friedland & Silverstein (1975).

Cell cultures

Primary cultures of porcine brain capillary endothelial cells were prepared as described (Audus & Borchardt, 1986) with the following modifications: cortical grey matter from six freshly obtained porcine brains was minced and incubated in Eagles minimum essential medium (MEM, Sigma, MO, U.S.A.) containing 0.5% dispase (Boehringer, Mannheim, Germany) for 2 h. Cerebral microvessels were obtained after centrifugation in MEM containing 13% dextran (Sigma). The microvessels were subsequently incubated in MEM containing 1 mg ml^{-1} collagenase/dispase (Boehringer) for 4.25 h. The resulting cell suspension was supplemented with 10% horse serum and was filtered through a 150 μm nylon mesh. Brain capillary endothelial cells were isolated on a continuous 50% Percoll gradient (Pharmacia, Uppsala, Sweden) C centrifugation: 1000 *g* for 10 min). Isolated endothelial cells were filtered through a 35 μm nylon mesh before seeding with a density of 10^5 cells cm^{-2} onto collagen/fibronectin (Boehringer) coated 24-well cell-culture plates (uptake assays) or with a density of 2×10^5 cells cm^{-2} onto polycarbonate membranes (transendothelial transport, see below). Cells were cultured under standard cell culture conditions (Audus & Borchardt, 1986) (cell culture medium: 45% MEM, 45% nutrient mixture F-12 ham (F12-HAM), 100 μg/ml^{-1} streptomycin, 100 μg ml^{-1} penicillin G, 100 μg ml^{-1} heparin, 13 mM $NaHCO_3$, 20 mM HEPES [all Sigma] containing 10% heat-inactivated horse serum [Gibco BRL, Switzerland]).

Immunostaining

For immunostaining, isolated brain endothelial cells were seeded onto chamber slides (Nunc, Naperville, Ill, U.S.A.) at a density of 2×10^5 cells cm^{-2}. Slides were pre-coated with poly-D-lysine (Sigma), collagen and fibronectin. At day 10 in culture, two days after the cells had reached confluence, cells were washed, fixed for 5 min in 4% paraformaldehyde and then permeabilized in 0.5% Triton X-100 for 5 min. Immunostaining for zona occludens protein 1 (ZO-1), a constituent protein of tight junctions, was carried out with an affinity purified rabbit anti ZO-1 antibody (Zymed, San Francisco, CA, U.S.A.) and a FITC conjugated secondary antibody (Dako, Glostrup, Denmark). Immunostaining for P-glycoprotein (P-gp) was carried out after fixation of the cells for 5 min in ice-cold acetone and permeabilization in 0.5% Triton X-100 for 5 min. A C219 monoclonal antibody (Mab) (Centocor, Malvern, PA, U.S.A.) was used in combination with a FITC conjugated secondary antibody (Dako). The cells, which were mounted in FluorSave (Calbiochem, CA, U.S.A.), were examined on a Zeiss Axiophot microscope equipped with a Zeiss Plan-Neofluar 40 × objective.

Vimentin was detected in tissue slices and cultured endothelial cells by a monoclonal mouse-anti-vimentin antibody (dilution 1:20 (Sigma, Taufkirchen, Germany)) using anti-mouse-IgG-FITC (1:100, Boehringer Mannheim, Germany) from rabbit as a secondary antibody. Isopropanol was used for tissue fixation. Von Willebrand factor was detected by a polyclonal antibody from rabbit which was kindly provided from Prof. G. Kurz, University Freiburg, Germany. Anti-IgG-POD from mice (Boehringer) served as a secondary antibody. Methanol was used for tissue fixation. All dilutions were done with PBS buffer containing 150 mM NaCl, 8.2 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4. Fluorescence microscopic examination of the stained tissue and cells was performed with a microscope IM-35 (Zeiss, Oberkochen, Germany) having an excitation wavelength between 450 and 495 nm and an emitted light filter >520 nm.

Immunodetection of P-gp

P-gp was detected by Western blot analysis using the monoclonal antibody C219 (Centocor). Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Mini-Protean II apparatus (Bio-Rad). To endothelial cell homogenates (1 mg protein ml^{-1}) one fifth volume sample buffer (10% glycerol, 5% SDS, 40 mM DTT, 0.00625% bromophenol blue, 62.5 mM Tris/HCl pH 6.8) was added. The samples were agitated 30 min at 25°C and loaded onto 6.5% acrylamide/bisacrylamide gels. After electrophoresis, proteins were transferred electrophoretically (2 h at a constant amperage of 2 mA cm^{-2}) to a 0.45 μm pore size nitrocellulose membrane using a Mini Trans-Blot cell (Bio-Rad). The transfer buffer contained 192 mM glycine, 25 mM Tris and 20% methanol. The membrane was blocked overnight at 4°C with 5% powdered skimmed milk in Tris-buffered saline (50 mM Tris and 150 mM NaCl) containing 0.3% Tween 20 (TBS-T). Washed membranes were incubated with Mab C219 (200 ng ml^{-1}) in TBS-T, 1% bovine serum albumin (BSA) and 0.05% NaN_3 for 2 h at 37°C. Washed membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1000) (Dako) in TBS-T containing 2% milk powder. Membranes were washed in TBS-T and P-gp was visualised by enhanced chemiluminescence detection (ECL-kit by Amersham, UK).

Uptake assays

Uptake assays were performed at 20°C in confluent monolayers of porcine brain capillary endothelial cells at day 10. Cells were grown in 24-well cell-culture plates. The surface area was 2 cm^2 per well. Cells were washed with transport buffer (mM: NaCl 122, KCl 3, $CaCl_2$ 1.4, $MgSO_4$ 1.2, D-glucose 5, HEPES 10, $NaHCO_3$ 25, K_2HPO_4 0.4, pH 7.4). The reaction was initiated by addition of 240 μl transport buffer containing 0.15 μCi of ^{3}H-isotope labelled tracer of the respective substrate, sufficient unlabelled substrate to bring the medium to the desired final concentration and 0.15 μCi of the extracellular markers [^{14}C]-sucrose or [^{14}C]-polyethylene glycol (PEG). For inhibition studies, cells were pre-incubated for 10 min with the respective inhibitor. Incubations were terminated after 5 min by rapidly removing the incubation medium followed by washing the cells using ice-cold transport buffer. Cells were then removed from the wells by incubation for 10 min in trypsin (0.25%) and were subsequently transferred to scintillation vials. Scintillation fluid was added, cells were solubilized overnight and the amount of radiolabelled substrate taken up by the cells was determined by scintillation counting.

Sublines of murine monocytic leukaemia P388 cells (Boesch *et al.*, 1991), one parental (Par-P388) and one multidrug-resistant (MDR-P388), were cultured as described by Boesch *et al.* (1991). Generation time of Par-P388 and MDR-P388 cells was 9 to 10 h. Viability of the cells was determined before and after the experiment by trypan-blue exclusion and was always greater than 95%. Total cell numbers were measured in a haemocytometer. To determine the initial velocity of uptake of M-6-G at a given concentration, 0.3 μCi of [^{3}H]-M-6-G and 0.3 μCi of the extracellular marker [^{14}C]-sucrose were added to 0.35 ml oxygenated transport buffer containing 1.0×10^6 cells followed by an incubation for 3 min in a shaker bath at 37°C. The final concentration of M-6-G was 0.68 mM. The incubation was terminated by placing two times 150 μl of the cell suspension in microcentrifuge tubes layered with 50 μl of 3 M KOH and 150 μl of silicone oil (1:1 (v/v) mixture of silicone oil type Ar20 and Ar200 (Wacker Chemie, München, Germany)) followed by rapid centrifugation in a tabletop microfuge (Hettich, Tuttlingen, Germany) capable of rapid acceleration. Upon centrifugation the cells passed through the oil and into

the KOH layer. Centrifugation tubes were then transferred to liquid nitrogen. The amount of substrate taken up was quantified by cutting the frozen centrifugation tube just above the KOH–oil interface and placing the tip of the tube with the pellet layer in a scintillation tube containing 500 μl Solutron tissue solubilizer (Kontron, Zürich, Switzerland). Samples were incubated overnight, sonicated in a water bath sonicator and scintillation fluid was added. The amount of radiolabelled substrate in the pellet layer was determined by scintillation counting.

Transendothelial transport

For the study of transendothelial transport, up to six polycarbonate membranes (snap-well system, Costar, MA, U.S.A.) with a confluent monolayer of porcine brain endothelial cells were mounted in a corresponding number of side-by-side diffusion cells (Costar). Both sides of the diffusion cells were filled with pre-warmed transport buffer. The whole system was kept at constant temperature (37°C) and was supplied with 5% CO_2/95% oxygen. At time $t=0$ the isotope-labelled compound to be studied was added to the apical donor-chamber. At defined time intervals samples were drawn from the acceptor chamber and were analysed by scintillation counting. The initial rate of transport was calculated from a linear regression. Permeability coefficients (P_{app}) were calculated according to: $P_{app} = dQ/dt \times 1/A/C_0$ (cm min^{-1}), where dQ/dt is the rate of translocation, A is the surface of the polycarbonate membrane and C_0 is the initial concentration of the labelled drug.

Statistical analysis

For statistical comparison, data were first tested for normal distribution by the Wilk-Shapiro test or Kolmogorov-Smirnov test, as appropriate. If normal distribution of data could not be rejected, groups were compared by analysis of variance (ANOVA), otherwise the Kruskal-Wallis test was applied. The level of significance was $P=0.05$. If this analysis revealed significant differences, pairwise comparisons between groups were performed by multiple two-sided unpaired t tests (normally distributed data) or Mann-Whitney tests (not normally distributed data) and P-values were adjusted by Bonferoni's

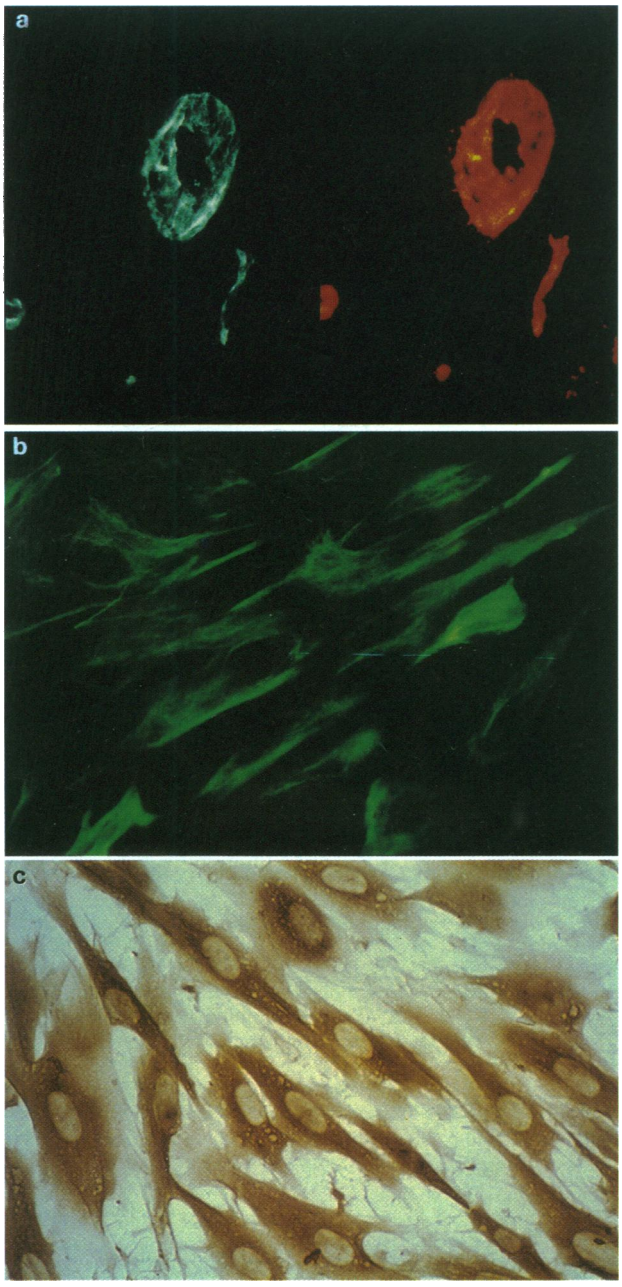

Figure 1 Immunofluorescence detection of cellular markers. Vimentin (a, left panel) and von Willebrand factor (a, right panel) were localized at brain capillaries in tissue slices (original magnification 240-fold, 85 × 100 μm section). Vimentin (b) and von Willebrand factor (c) were also present in cultured brain capillary endothelial cells at day 10 in culture (original magnification 240-fold, 40 × 60 μm section.

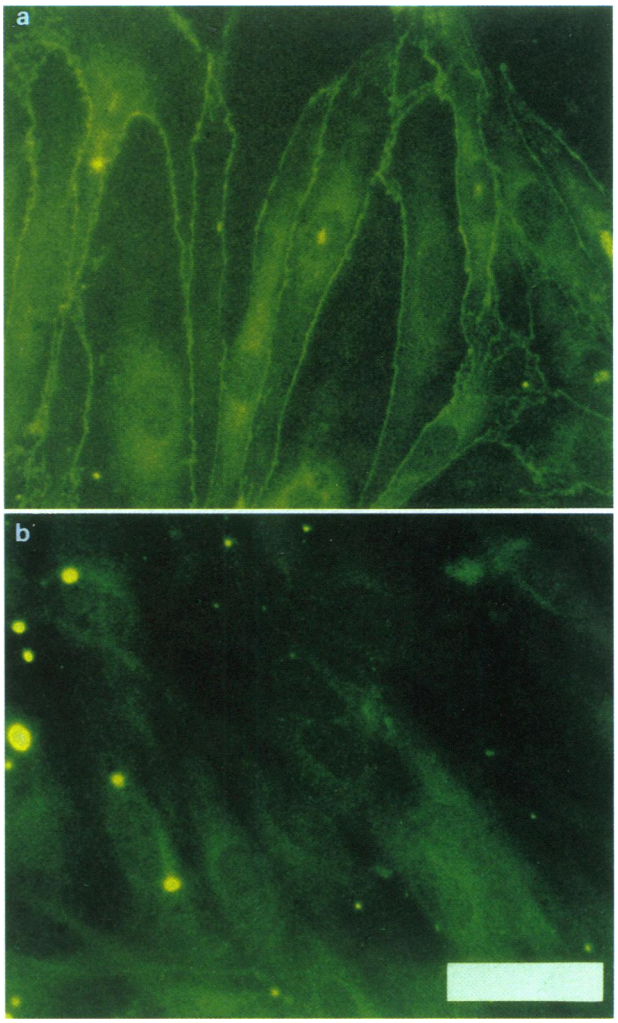

Figure 2 Immunofluorescent localization of the tight junction-associated protein ZO-1 in primary cultures of porcine brain capillary endothelial cells at day 10. (a): Immunostaining for ZO-1; (b): control using rabbit serum in combination with the secondary antibody. Bar = 30 μm.

correction. Statistical analysis was performed using the SPSS for Windows software (1994).

Results

Characterization of cell cultures

Primary cultures of porcine brain capillary endothelial cells were used as an *in vitro* cell culture model of the blood-brain barrier. Biochemical characterization of cultured endothelial cells revealed a pronounced enrichment in enzymatic activities of marker enzymes (alkaline phosphatase: 549.8±34.5 nmol mg^{-1} min^{-1} and angiotensin converting enzyme: 29.6±4.4 nmol mg^{-1} min^{-1}) as compared to whole brain homogenates (alkaline phosphatase: 20.6±2.2 nmol mg^{-1} min^{-1} and angiotensin converting enzyme: 0.23±0.009 nmol mg^{-1} min^{-1}. Values represent means±s.e.mean of at least 5 determinations). As demonstrated by immunostaining, cultured endothelial cells express the endothelial cell specific marker von Willebrand factor (factor VIII-related antigen) (Figure 1), vimentin (Figure 1) as well as the tight junction associated protein ZO-1 (Figure 2). In addition, cultured cells accumulated rhodamine-labelled acetylated low-density lipoprotein (data not shown). Functional characterization of the cultured cells was done by probing for active transport of uncharged amino acids (leucine, phenylalanine) by the large neutral amino acid carrier system (L-system) (Pardridge & Oldendorf, 1975). The Michaelis-Menten constant (K_M) for uptake of leucine was 19 μM. Uptake of leucine could be competitively inhibited by phenylalanine (K_I = 13 μM). Transendothelial transport of leucine across endothelial cell monolayers grown on polycarbonate filter inserts was saturable and showed Michaelis-Menten type kinetics (K_M = 1.2 mM).

Transport of M-6-G across brain capillary endothelial cells

Determination of the octanol to buffer partition coefficient of M-6-G revealed a lipophilicity of this compound which is higher than the one of the extracellular marker sucrose but considerably lower than the values for morphine or propranolol (Table 1). On the assumption that M-6-G penetrates the blood-brain barrier by passive diffusion only, the permeability coefficient of M-6-G could be expected to range between the coefficient of sucrose and morphine. However, transport experiments (Table 1) revealed a rate of penetration of M-6-G through confluent monolayers of brain endothelial cells, which was comparable to the value for sucrose. This finding was confirmed by experiments, where time-dependent uptake of M-6-G by brain endothelial cells was compared to the uptake of sucrose, morphine and propranolol (Figure 3). Here again, uptake of M-6-G was marginal and not significantly different from the extracellular marker sucrose. Compared to M-6-G, a significantly ($P<0.001$) larger amount of morphine and propranolol was taken up during an incubation period of 50 min. Although the kinetics of uptake over time were different between morphine and propranolol, a comparable fraction of the dose was taken up after 50 min.

P-glycoprotein

By immunostaining, expression of the multidrug resistance transporter P-gp by our cultured porcine brain capillary endothelial cells was shown (Figure 4). Since recently a cross-reactivity of the monoclonal antibody C219 in bovine and rat isolated brain capillaries with a 190 kD protein was reported (Beaulieu *et al.*, 1995), this finding was corroborated by Western blot analysis of porcine brain capillary endothelial cells (Figure 5). Two sublines of murine monocytic leukaemia P388 cells, one parental (Par-P388) and one multidrug-resistant (MDR-P388), were used as a control (Boesch *et al.*, 1991). Using the monoclonal antibody C219 only one protein with apparent molecular mass of 150 kDa was immunodetected in MDR-P388 cells and in porcine brain capillary endothelial cells. No binding of antibody could be detected in Par-P388 cells. In addition, no cross-reactivity of the antibody with our cells was observed. Uptake assays (Figure 6), which were performed in the presence or absence of the known inhibitors of P-gp verapamil and vincristine, demonstrated that uptake of M-6-G into brain endothelial cells can be enhanced significantly ($P<0.0001$) in presence of these multidrug-resistance modifiers. Uptake of the extracellular marker polyethylene glycol was not affected by these compounds. The finding that M-6-G may serve as a substrate for P-gp was confirmed in uptake assays performed using parental murine monocytic leukaemia P388 cells (Par-P388) and multidrug-resistant P388 cells (MDR-P388) (see above). Uptake of M-6-G into drug-

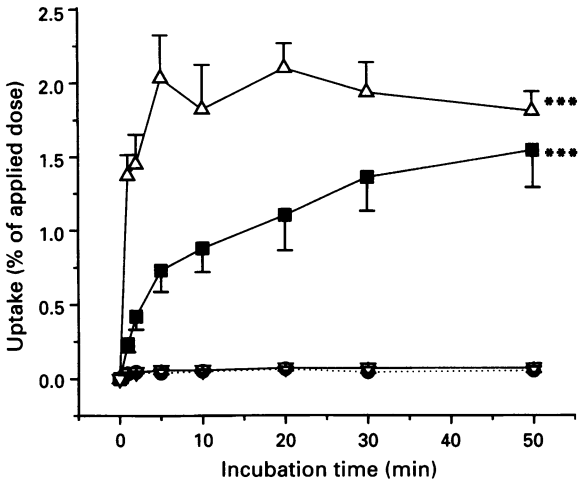

Figure 3 Time dependent uptake of [^{3}H]-morphine-6-glucuronide (● and dashed line, 135 μM, [^{3}H]-morphine (■, 150 μM) and [^{3}H]-propranolol (△, 150 μM). [^{14}C]-sucrose (▽) was used as an extracellular marker. Data points are means±s.e.mean, n=27. Significantly different ***($P<0.001$; multiple two-sided Mann-Whitney tests with Bonferroni's correction) from cumulative [^{14}C]-sucrose and [^{3}H]-morphine-6-glucuronide uptake over 50 min. No statistically significant differences were observed between cumulative [^{14}C]-sucrose and [^{3}H]-morphine-6-glucuronide as well as between cumulative [^{3}H]-morphine and [^{3}H]-propranolol uptake.

Table 1 Partition coefficients and permeability coefficients through brain capillary endothelial cell monolayers

Compound	*Partition coefficient*	*Permeability coefficient* ($\times 10^{-3}$ cm min^{-1})
[^{14}C]-sucrose	0.0011±0.0003 (n=3)	2.32±0.18 (n=11)
[^{3}H]-morphine-6-glucuronide	0.0045±0.0014 (n=3)	2.28±0.20 (n=4)
[^{3}H]-morphine	0.3767±0.0071 (n=3)	3.28±0.25 (n=3)
[^{3}H]-propranolol	12.2091±0.6802 (n=3)	7.35±0.36 (n=4)

Values represent means ±s.e.mean.

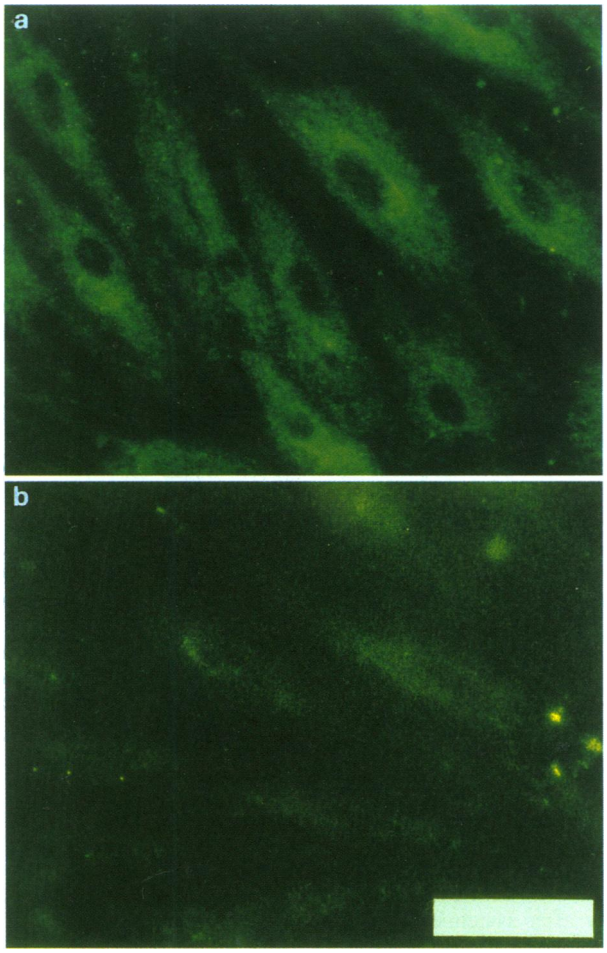

Figure 4 Immunodetection of P-glycoprotein in porcine brain capillary endothelial cells. (a) Immunostaining for P-glycoprotein using the monoclonal antibody C219; (b) secondary antibody only. Bar = 30 μm.

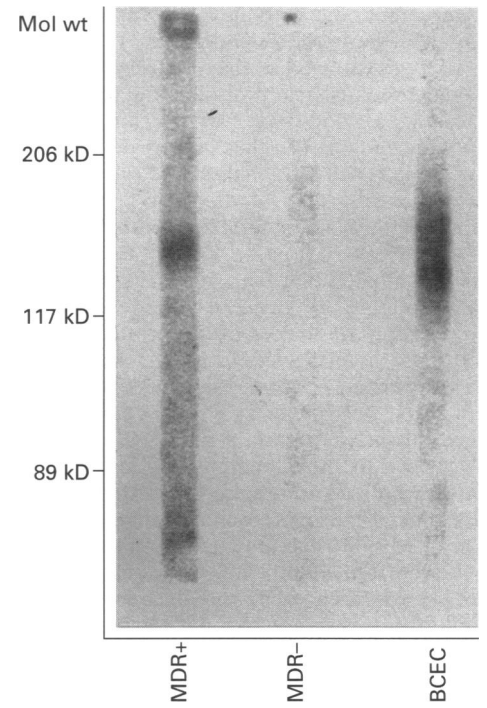

Figure 5 Western blot analysis of P-glycoprotein from porcine brain capillary endothelial cells (BCEC). As a control, Par-P388 cells (MDR−) and MDR-P388 cells (MDR+) expressing the MDR phenotype were used. A protein with apparent molecular mass of 150 kD was immunodetected in MDR+ and BCEC by the monoclonal antibody C219. Molecular weight standards are myosin (206 kDa), β-galactosidase (117 kDa) and bovine serum albumin (89 kDa).

sensitive cells (Par-P388, uptake was 3.04 ± 0.09 pmol M-6-G min^{-1} per 10^6 cells, $n = 10$) was higher when compared to uptake into cells over-expressing P-gp (MDR-P388, 2.32 ± 0.26 pmol M-6-G min^{-1} per 10^6 cells, $n = 10$. Values represent means ± s.e.mean). This difference was significant by analysis of variance ($P < 0.05$).

Discussion

Biochemical characterization of porcine brain capillary endothelial cells revealed a marked enrichment in enzymatic activity of alkaline phosphatase (56 fold) and angiotensin converting enzyme (230 fold) as compared to whole brain tissue. The activity of these marker enzymes decreased only slightly in the cultured cells as compared to the freshly isolated cells. By immunostaining, the presence of factor VIII, vimentin and the tight junction associated protein ZO-1 was shown. Thus the identity and integrity of our primary cultures of brain capillary endothelial cells was confirmed. Functional characterization of endothelial cell monolayers revealed the intact function of the carrier system responsible for transport of neutral amino acids (Hughes & Lantos, 1989). The Michaelis-Menten constant determined for uptake of leucine ($K_M = 19$ μM) was similar to reported values determined for rat brain endothelial cells ($K_M = 83$ μM) (Hughes & Lantos, 1989). Uptake of leucine showed competitive inhibition by phenylalanine. Transendothelial transport of leucine across endothelial cell monolayers was saturable and showed Michaelis-Menten type kinetics. The K_M of 1.2 mM for transendothelial transport

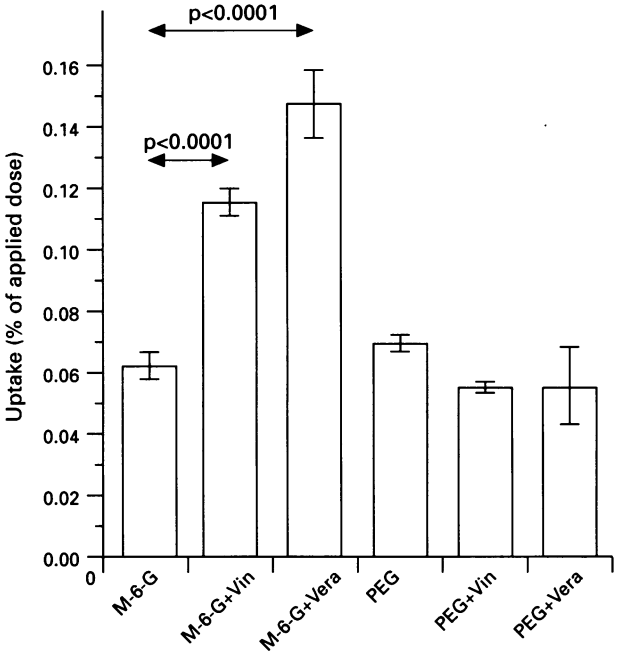

Figure 6 Uptake of [^{3}H]-morphine-6-glucuronide (135 μM) with or without inhibitors of P-glycoprotein (Vin = 100 μM vinblastine, Vera = 100 μM verapamil) by porcine brain capillary endothelial cells. As a control, uptake of the extracellular marker polyethylene glycol (PEG) in presence or absence of inhibitor was determined in a separate set of experiments. Data points represent means ± s.e.mean, $n = 25$, 35, 23, 16, 32 or 5, from left to right. Statistical comparisons: multiple two-sided Mann-Whitney tests with Bonferroni's correction.

of leucine was comparable with the K_M (0.15–0.16 mM) for the L-system *in vivo* (Pardridge & Oldendorf, 1975). From these experiments it was concluded that our *in vitro* blood-brain barrier model represents an appropriate tool to study transport processes across the blood-brain barrier. Thus, our model is in accordance with findings of other groups demonstrating preservation of important functional characteristics of brain capillary endothelial cells after isolation and appropriate culture conditions (Pardridge *et al.*, 1990; Dehouck *et al.*, 1992; Chesne *et al.*, 1993).

It has been estimated that the cerebrovascular permeability of an agent is roughly proportional to its lipid solubility. Exceptions to this rule have been demonstrated and occur for agents that share a facilitated transport mechanism at the cerebral capillaries, i.e. glucose, large neutral L-amino acids and related drugs, electrolytes as well as compounds of large molecular weight such as certain peptides (Bradbury, 1992). In the case of M-6-G, the lipid solubility of this compound does not correlate with its transport properties. Uptake and transendothelial transport assays revealed an unexpectedly low permeability of M-6-G. The presence of an active transporter extruding M-6-G from endothelial cells would offer a possible explanation for this phenomenon. P-glycoprotein, which confers the multidrug resistance (MDR) phenotype to tumour cells, is such a transport system. Drugs are proposed to be actively extruded from the cells using energy derived from P-glycoprotein (P-gp) mediated ATP hydrolysis (Endicott & Ling, 1989). P-gp has a broad substrate specificity. Agents which may serve as substrates for P-gp include antitumour alkaloids such as vinblastine, the calcium antagonist verapamil (Spoelstra *et al.*, 1994) or cyclosporin A, a cyclic peptide of 11 amino acids (Twentyman, 1992). P-glycoprotein is expressed in several normal tissues including, among others, liver, kidney, colon (Thiebaut *et al.*, 1987) and the endothelial cells of the blood-brain barrier (Endicott & Ling, 1989; Tatsuta *et al.*, 1992). P-gp was localized at the luminal side of capillary endothelial cells in both gray matter of the brain and primary cultured bovine brain capillary endothelial cells (Tsuji *et al.*, 1992). In another study, P-gp, recognised by the murine monoclonal antibody MRK 16, was strictly confined to the luminal surface of the endothelial cells which comprise the capillary vessels of the brain and the spinal cord. In addition, the findings implied that the blood-brain barrier is anatomically characterized by the presence of intercellular tight junctions between continuous nonfenestrated endothelial cells (Sugawara *et al.*, 1990). They are in agreement with our results demonstrating the presence of P-gp in porcine brain capillary endothelial cells by immunostaining and Western-blot analysis.

Two lines of evidence support our view that M-6-G may serve as a substrate for P-gp: first, uptake of M-6-G by brain capillary endothelial cells is enhanced significantly in the presence of the known multidrug-resistance reversers, verapamil and vincristine. Second, murine tumour cells overexpressing P-gp accumulate less M-6-G. Thus, using a different cellular system, our observation was confirmed. Taken altogether, these observations provide strong evidence for the presence of a specific efflux system for M-6-G which may be related to P-gp. As discussed above, P-gp is present at the blood-brain barrier and is also expressed by our cellular system. In addition, using multidrug-resistant Chinese hamster ovary cells, it was recently shown (Callaghan & Riordan, 1993) that these cells accumulate significantly less morphine and related narcotic analgesics suggesting that, at least in this cellular system, morphine is a substrate for P-glycoprotein. Furthermore, a glucuronide conjugate of oestradiol, 17β-oestradiol glucuronide, is a physiological substrate for P-gp in hepatocytes (Gosland *et al.*, 1993).

Although we were able to provide evidence for an efflux system for M-6-G in brain capillary endothelial cells, the molecular mechanism of M-6-G penetration into the cells remains unclear. Inhibitors of P-gp stimulate significantly the uptake of M-6-G. Under these conditions, the overall uptake still remains in the range of the uptake of the extracellular marker polyethylene glycol (Figure 5). Our data might suggest that the mechanism of M-6-G uptake is either by simple diffusion or by a carrier system which has a very weak affinity for M-6-G. However, in view of the pronounced pharmacological activity of this compound (Paul *et al.*, 1989) even small amounts of M-6-G penetrating the blood-brain barrier are far from being negligible.

From our experiments we conclude that M-6-G may serve as a substrate for P-glycoprotein. Thus, uptake of M-6-G into nonfenestrated brain capillary endothelial cells and thereby penetration of the brain by M-6-G may be modulated by P-gp. The findings do not exclude a significant uptake of M-6-G through fenestrated capillaries as found in peri-ventricular structures. However, preliminary results of M-6-G brain uptake after systemic administration in rats indicate a reduced penetration compared to morphine similar to that observed in the present *in vitro* study (Bickel *et al.*, 1996). Since M-6-G is a pharmacologically active metabolite of morphine, this finding may offer an explanation for the great inter-individual variation in the effective analgesic dose of morphine in man. If morphine is co-administrated with drugs used in chemotherapy, which are often known substrates of P-gp, this effect could be further amplified.

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