Pharm Res (2015) 32:1072–1083
DOI 10.1007/s11095-014-1519-8

RESEARCH PAPER

Permeability of PEGylated Immunoarsonoliposomes Through *In Vitro* Blood Brain Barrier-Medulloblastoma Co-culture Models for Brain Tumor Therapy

Abdulghani Al-Shehri • Marco E. Favretto • Panayiotis V. Ioannou • Ignacio A. Romero • Pierre-Olivier Couraud • Babette Barbash Weksler • Terry L. Parker • Paraskevi Kallinteri

Received: 11 May 2014 / Accepted: 12 September 2014 / Published online: 19 September 2014

ABSTRACT

Purpose Owing to restricted access of pharmacological agents into the brain due to blood brain barrier (BBB) there is a need: 1. to develop a more representative 3-D-co-culture model of tumor-BBB interaction to investigate drug and nanoparticle transport into the brain for diagnostic and therapeutic evaluation. 2. to address the lack of new alternative methods to animal testing according to replacement-reduction-refinement principles. In this work, *in vitro* BBB-medulloblastoma 3-D-co-culture models were established using immortalized human primary brain endothelial cells (hCMEC/D3).

Methods hCMEC/D3 cells were cultured in presence and in absence of two human medulloblastoma cell lines on Transwell membranes. *In vitro* models were characterized for BBB formation, zonula occludens-1 expression and permeability to dextran. Transferrin receptors (Tfr) expressed on hCMEC/D3 were exploited to facilitate arsonoliposome (ARL) permeability through the BBB to the tumor by covalently attaching an antibody specific to human Tfr. The effect of anticancer ARLs on hCMEC/D3 was assessed.

Results *In vitro* BBB and BBB-tumor co-culture models were established successfully. BBB permeability was affected by the presence of tumor aggregates as suggested by increased permeability of ARLs. There was a 6-fold and 8-fold increase in anti-Tfr-ARL uptake into VC312R and BBB-DAOY co-culture models, respectively, compared to plain ARLs.

Conclusion The three-dimensional models might be appropriate models to study the transport of various drugs and nanocarriers (liposomes and immunoarsonoliposomes) through the healthy and diseased BBB. The immunoarsonoliposomes can be potentially used as anticancer agents due to good tolerance of the *in vitro* BBB model to their toxic effect.

KEY WORDS arsonolipids · blood brain barrier cancer model · cell aggregates · hCMEC/D3 cells · transferrin receptors

ABBREVIATIONS

Anti-Tfr-ARLs	Antibody (against transferrin receptor) bearing PEGylated arsonoliposomes
ARL	Arsonoliposomes
ATO	Arsenic trioxide
EDC	N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide
FCS	Fetal calf serum
NGS	Normal goat serum
PEG-ARLs	PEGylated arsonoliposomes
PFA	Paraformaldehyde
PI	Propidium iodide
S-NHS	N-Hydroxysulfosuccinimide sodium salt
TEER	Transendothelial electronic resistance

Electronic supplementary material The online version of this article (doi:10.1007/s11095-014-1519-8) contains supplementary material, which is available to authorized users.

A. Al-Shehri · T. L. Parker
School of Biomedical Sciences, University of Nottingham, Nottingham NG7 2RD, UK

M. E. Favretto · P. Kallinteri (✉)
Medway School of Pharmacy, Universities of Kent/Greenwich, Central Avenue, Chatham Maritime, ME4 4TB Kent, UK
e-mail: pkallinteri@gmail.com

P. V. Ioannou
Department of Chemistry, University of Patras, Rio, Patras 26504, Greece

I. A. Romero
Department of Biological Sciences, Faculty of Science, The Open University, Walton Hall, Milton Keynes MK7 6AA, UK

P.-O. Couraud
Université Paris Descartes, INSERM, U567, 75014 Paris, France

B. B. Weksler
Weill Cornell Medical College, 525 East 68th Street, Payson Pavilion, 3, New York, New York 10065, USA

Tfr	Transferrin receptor
ZO-1	Zonula occludens

INTRODUCTION

Brain homeostasis is maintained by the blood brain barrier (BBB) which is the most restrictive barrier in our body due to the lack of paracellular pathway of solutes (1). Only highly lipophilic molecules with molecular weight of less than 400 Da can cross freely the BBB. The majority of drugs developed to target the CNS cannot cross the BBB *via* diffusion but only *via* a transporter in case they are substrates for the large number of such transporters expressed on the endothelial cells of the barrier (1). Drug design and development as well as physiology, pharmacology and pathology of the BBB will be better understood by creating representative *in vitro* BBB models. Many attempts have been made to produce reliable *in vitro* models but there are always some limitations such as the cost, time consuming and of short life construct of a model using primary bovine mainly endothelial cells, or models characterized by high paracellular permeability (2). Therefore, human immortalized cell lines have been developed: BB19 (3), NKIM-6 (4) and hCMEC/D3 (5). BB19 use is limited by its high permeability and NKIM-6 haven't been studied and characterised extensively enough to be considered as a reference cell line in the development of blood brain barrier models.

However, hCMEC/D3 can be used in Transwell and dynamic *in vitro* blood brain barrier models. In recent studies, it has been demonstrated that hCMEC/D3 cells retain transporter expression and functionality, as well as low paracellular permeability (6). Moreover, it has been shown (7) that they exerted the same transendothelial electrical resistance in presence or absence of support cells.

In order to enhance the permeability of drug molecules across the BBB, nanoparticles and liposomes have been used (8). Liposomes loaded with anticancer agents (Doxil®) are in clinical use administered either intravenously or intrathecally (9). The accumulation of liposomes in the tumor site is achieved due to the leaky tumor vasculature, a phenomenon known as enhanced permeation and retention effect (10). More effective nanocarrier accumulation has been reported by exploiting transporters such as insulin or transferrin (8). This is achieved by attaching monoclonal antibodies against the particular receptors on the liposome surface. Also, it is known that tumor cells overexpress transferrin receptors. Therefore, by using anti-transferrin receptor conjugated liposomes would enable the traverse of liposomes across the BBB and also the higher accumulation of liposomes in the tumor cells.

Moreover, arsenic compounds, in the form of arsenic trioxide (ATO), have been used as a treatment of acute promyelocytic leukemia. Its use has been gradually discontinued over the years because of the low selectivity of these compounds, which results in a high toxicity against both tumor and healthy cells (11). Thus, there has been considerable research on methods to enhance the efficacy of arsenic while reducing its toxicity against the normal cells. Liposomes encapsulating ATO have been prepared (12) and they were proven insufficient in retaining arsenic trioxide. However, it was shown that arsonoliposomes decrease significantly the viability of several types of cancer cells while being ineffective towards normal cells under the same exposure conditions (13,14).

Therefore, the objectives of this work were to construct a 3D *in vitro* co-culture model of BBB-tumor in order to use it for screening purposes of newly developed drugs and nanocarriers. Also, anti-Tfr-arsonoliposomes were used to validate the models as well as to assess the toxicity and permeability across the BBB-tumor co-culture models of plain and targeted arsonoliposomes.

Materials and Methods

Cell Culture

hCMEC/D3 cells used for the BBB model (passage 27–34) were kindly provided by Dr. Ignacio A. Romero (5). hCMEC/D3 were cultured as monolayers in EGM-2 medium (Lonza, UK) consisting of EBM-2 basal medium with addition of basic Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Insulin-like Growth Factor-1 (IGF-1), ascorbic acid, hydrocortisone, gentamicin, and 2.5% Fetal Bovine Serum (FBS) (5). Cells were grown in flasks pre-coated with rat collagen type I (BD Biosciences, UK) (1 mg/ml) and maintained in a humidified incubator at 37°C and 5% CO_2. Culture medium was renewed every 2 to 3 days.

Human medulloblastoma cell line (VC312R) was provided by Professor Geoff Pilkington, University of Portsmouth, UK. Human medulloblastoma cell line (DAOY) was purchased from ATCC (American Type Culture Collection, Manassas, USA). Both, VC312R and DAOY cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM), with 15% FBS and 200 mM L-glutamine at 37°C and 5% CO_2. Unless otherwise indicated, all culture reagents were supplied from Invitrogen Life Technologies Ltd. (Paisley, UK).

Development of BBB and BBB-tumor Co-culture *In Vitro* Models

hCMEC/D3 cells were seeded on the luminal side of an insert (polyester membrane, 12 mm diameter, 0.4 μm pore size, Transwell Corning, Costar, UK) pre-coated with collagen I

(100 µl, 0.1 mg/µl; BD Biosciences, UK) and fibronectin (100 µl, 20 µg/ml, Sigma-Aldrich, UK) at a density of 1.2×10^5 cells per filter.

For the co-culture of endothelial cells and tumor aggregates, a fresh coating of fibronectin and collagen I was applied on both insert sides. The insert was inverted and 4×10^4 either DAOY or VC312R cells in the form of aggregates in 100 ml of medium (DMEM with 15% FBS and 200 mM L-glutamine) were seeded on the abluminal (lower side) of the membrane insert, which was then placed in a six well plate. The cells were incubated for 4 h at 37°C and 5% CO_2 and the filter was then returned to its original 12-well plate. Then, 1.2×10^5 hCMEC/D3 cells per filter were seeded on the insert's luminal side, and the co-culture was incubated for 7 days with medium replacement every 2 days.

Characterization of BBB

The formation of tight junctions between the hCMEC/D3 cells was investigated by estimating the presence of Zonula Occludens 1 (using TEM and immunocytochemistry) and assessing dextran permeability and TEER.

Transmission Electron Microscopy (TEM)

hCMEC/D3 cells grown on the inserts, or co-cultured with DAOY or VC312R cells for 6 to 7 days were fixed in 3% glutaraldehyde made up in 0.1 M cacodylate buffer. The filter was washed with cacodylate buffer and post-fixed with 1% osmium tetroxide for 30 min. The cells were dehydrated in an ethanol gradient (from 50%, 70%, 90% to 100%) (2×5 min). Then, the cells were placed successively in 100% acetone, 1:3 acetone: resin mixture, 1:1 acetone: resin for 1 h, pure resin (3×1 h), and embedded in a pure resin at 60°C for 48 h to allow polymerization of the resin. The polymerized blocks were cut into semi-thin (120 nm thick) sections using an ultra-microtome (Cut 4060, Slee Germany). The semi-thin sections were stained with an alkaline toluidine blue solution, and the ultra-thin sections were placed on formvar-coated copper grids, and finally stained with uranyl acetate and lead citrate. Samples were imaged under Transmission Electron Microscopy (TEM) (Tecnai BioTwin, FEI, USA). All reagents used for TEM were from Agar Scientific Ltd. (Essex, UK).

Zonula Occludens Protein (Z0-) Expression

hCMEC/D3 cells grown to confluence on filters in the absence or presence of medulloblastoma cell lines were fixed with 4% (w/v) paraformaldehyde in PBS. After washing twice with PBS, the cells were permeabilized and non-specific binding sites were blocked with PBS containing 0.25% Triton X-100 and 5% normal goat serum (NGS) at room temperature (RT). Polyclonal anti-Zonula occludens protein antibody (ZO-1) (Zymed antibodies, Invitrogen) in 2% NGS diluted in PBS 1:50 was added to the endothelial cells, which were then incubated overnight at 4°C. After washing with PBS, the filter membrane with the cells was cut out of the inserts and transferred into a multiwell plate. Cells were incubated with a 1:400 dilution of goat anti-rabbit Alexa Fluor 488 labeled secondary antibodies at RT for 1 h in the dark. Finally, the cells were washed three times with PBS, and the filter membrane with the stained cells were put on a slide mounted with DAPI (Vector Laboratories) and observed on an epifluorescent microscope (LEICA, DMRB).

Assessment of Dextran Permeability

The cell culture inserts were transferred to a 12-well plate containing 1.5 ml DMEM without phenol red (Invitrogen) supplemented with 10 mM HEPES (Sigma) and 10% FCS (Invitrogen). The culture medium in the filter's luminal side was replaced by 0.5 ml of the same solution containing 1 mg/ml fluorescently labelled dextran (MW: 4 kDa, 40 kDa and 70 KDa; Sigma-Aldrich, UK). The inserts were transferred to new wells containing 1.5 ml DMEM without phenol red supplemented with 10 mM HEPES and 10% FCS at 10, 20, 30, 40 and 50 min. FITC-dextran permeability through the filters was estimated by measuring the fluorescence (λ_{exc}.: 490 nm, λ_{em}: 520 nm) in the abluminal compartment using a fluorescence microplate reader (RF-5000, Shimadzu, Japan). Dextran permeability through the coated with collagen and fibronectin filter in absence of cells was used as a control. Permeability coefficient (P_e) values of the endothelial monolayer were calculated by the following equations:

$$\frac{1}{PS}=\frac{1}{me}-\frac{1}{mf} \tag{1}$$

and

$$Pe=\frac{PS}{S} \tag{2}$$

where *PS* is the permeability-surface area product (ml/min), *S* is the surface area of the filter (cm^2), and *me* and *mf* are the volumes cleared *vs.* time (ml/min) corresponding to endothelial cells on filters and to empty filters, respectively (Eq. 1) (15). The experiment was repeated three times, for three samples each time.

[^{14}C]-sucrose (Amersham, 0.6 µC/ml) permeability through the *in vitro* BBB in absence of the tumor cells was measured using the same exact protocol as described above.

TEER measurements were performed on both hCMEC/D3 monolayer and the co-culture with medulloblastoma to determine the tightness of the cellular barrier. Samples were placed in an Endohm-12 chamber, which was connected to a voltameter (Millicell ERS, Millipore). First, TEER measurement was conducted on blank Transwell filter inserts. The TEER value for blank filter was subtracted from those measurements where the filter carried cells either as monoculture or co-culture form.

Transferrin Receptor Expression on hCMEC/D3 Cells

hCMEC/D3 cells were grown to sub-confluency on coverslips previously coated with type I collagen in the conditions described above and fixed with 4% PFA in PBS. After washing twice with PBS, the cells were permeabilized and non-specific binding sites were blocked with PBS containing 0.25% Triton X-100 and 5% goat serum (NGS) at room temperature. Monoclonal antibody against transferrin receptor in 2% NGS diluted PBS (1:100) was added to the endothelial cells which were incubated overnight at 4°C. After washing with PBS, cells were incubated with a 1:400 dilution of goat anti-mouse Alexa Fluor 488 labeled secondary antibodies at room temperature for 1 h in the dark. Finally, the cells were washed three times with PBS, and the coverslips with the stained cells were mounted with DAPI containing mounting medium and observed under a Leica epi-fluorescent microscope.

Liposome Preparation and Characterization

PEGylated small unilamellar arsonoliposomes (PEG-ARLs) were prepared with distearoyl-phosphatidylcholine (DSPC) (Lipoid, Germany), arsonolipid (C_{16}-As; synthesised by Panayiotis V. Ioannou, Greece), cholesterol (Chol) (Sigma-Aldrich, UK) at the molar ratio of 12:8:10 and 8% molar ratio DSPE-mPEG2000 (Avanti, USA) according to Favretto *et al.* (14). Fluorescently labeled PEGylated small unilamellar arsonoliposomes were prepared in the same way by adding the fluorescent dye (Bodipy-DHPE; Invitrogen, UK or Rhod-DHPE; Sigma-Aldrich, UK) in the initial lipid mixture at 0.2% molar ratio of the total lipids. After preparation, liposomes were passed through a Sephadex G50 column (1 mm× 40 mm) to separate the free dye.

Antibody bearing arsonoliposomes (anti-Tfr-ARLs) were prepared in the same way as described above but the PEGylated lipids used were a mixture of PEG2000-DSPE and NH_2-PEG2000-DSPE in a molar ratio 4:1. The C-terminus of the monoclonal antibody (500 μg/750 μl) against the human transferrin receptor was activated using S-NHS (0.6 mg/ml of antibody; 0.45 mg) and EDC (0.4 mg/ml of antibody; 0.3 mg), by stirring for 30 min, at room temperature (16). Finally, liposomes and antibody were combined in a ratio of 75 μg antibody/μmol of phospholipid. The mixture was kept under stirring for 2 h, at room temperature. The conjugate was separated using a Sepharose 4B-CL column with PBS 7.4 as eluent. The amount of antibody conjugated to the arsonoliposomes was quantified using Bradford assay (17).

Radiolabelled small unilamellar arsonoliposomes, either plain or antibody bearing, were prepared following the same protocol as above, by adding tritiated cholesterol (1 μCi/mg of lipid) in the initial lipid dispersion. Liposomes were passed through a PD-10 column (GE Healthcare, UK) to separate any free radioactive cholesterol.

PEGylated DSPC/Chol small unilamellar liposomes were used as negative control to the arsonoliposome action and made using the thin film method (14).

Lipid concentration was estimated using Stewart assay (18). Liposome size distribution was measured at 25°C using photon correlation spectroscopy at a fixed angle of 90° and zeta potential was measured using Laser Doppler Anemometry (Malvern Instruments Ltd., Malvern, UK).

Effect of Arsonoliposomes on Endothelial Cells

Cytotoxicity Studies

Cell viability of endothelial cells was evaluated using MTT assay. Briefly, cells were seeded at a concentration of 5×10^4 cells/ml/well in 24-well plates and were incubated with either PEG-DSPC/Chol or PEG-ARLs at increasing concentrations (20 μg/ml to 1,000 μg/ml) for a time period of 1–4 days. Non-treated cells were used as negative control, while DMSO (0.5 ml/well) was used as positive control for cell death. The MTT protocol used was exactly the same as that in Favretto *et al.* (14).

Early Apoptosis Studies

Endothelial cells were seeded in a 24-well plate at the concentration of 2×10^4 cells/ml/well and incubated with arsonoliposomes (200 μg/10^6 cells) in presence of 1% serum at 37°C for 2, 5 and 24 h. TNF-α (50 ng/ml) was added as positive control for apoptosis induction and PEG-DSPC/Chol plain liposomes at 200 μg/10^6 cells were used as negative control. Non-treated cells were used to evaluate the presence of false positive. After incubation, cells were washed with cold PBS and re-suspended in 200 μl buffer consisted of 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ (Annexin binding buffer). After the addition of 10 μl of Alexa Fluor 488 annexin V (Invitrogen, UK), the cells were incubated at room temperature, in the dark, for 15 min. Then, the cells were washed with Annexin binding buffer and

the coverslips mounted on the slides using a PI-containing mounting medium to stain the dead or apoptotic nuclei. Finally, the samples were observed under a Leica epi-fluorescent microscope.

Permeability of PEG-ARLs and Anti-Tfr-ARLs Through the *In-Vitro* BBB and BBB-Tumor Co-culture Models

To measure the permeability of both, PEG-ARLs and anti-Tfr-ARLs through the *in-vitro* BBB and BBB-tumor co-culture models, hCMEC/D3 cells were cultured at a density of 1.2×10^5 cells per filter to confluence on the luminal side of a Transwell insert pre-coated with collagen IV and fibronectin. For co-cultures, medulloblastoma cell lines were grown on the abluminal side of the insert at a density of 4×10^4 cells. Permeability assays were carried out after 6–7 days. After removing cell culture medium from both compartments, the inserts were washed with pre-warmed PBS. The cell culture inserts were then transferred to a 12 well plate containing 1.5 ml DMEM without phenol red supplemented with 10% FCS. The culture medium in the upper compartment was replaced by 0.5 ml of the same medium containing tritiated either PEG-ARLs or anti-Tfr-ARLs at a lipid concentration of 400 μg/ml. After 2 and 5 h, the inserts were then transferred to a new well containing 1.5 ml DMEM without phenol red supplemented with 10 mM HEPES and 10% FCS. Liposome movement was detected by measuring the radioactivity, in terms of disintegrations per minute, in the different compartments of the model: luminal (endothelial cell side), filter, tumor (if present) and abluminal (well side). Empty filters, pre-coated with rat-tail collagen and fibronectin, in the absence of cells, were used as controls. Apparent permeability was calculated using the equation

$$Papp = \frac{dC}{dt} \times \frac{Vr}{A \times C_0} \tag{3}$$

P_{app}: apparent permeability, dC/dt: change in the radiochemical concentration (dpm/mL/s) in the receiver chamber, Vr: volume in the receiver chamber, A: surface area of the cell monolayers, C_0: initial concentration in the donor chamber (dpm/mL) (19).

Statistical Evaluation

ANOVA and *t*-test were used for the statistical evaluation of the results presented in the current work.

RESULTS

Development of BBB and BBB-Tumor Co-culture *In Vitro* Models

Characterization

All *in-vitro* models were characterized in terms of BBB elements such as zonula occludens protein expression, paracellular permeability using hydrophilic markers of various sizes and transferrin receptor expression by the endothelial cells in absence and in presence of the medulloblastoma cell lines. [^{14}C]-sucrose (M.W. 342 Da) permeability through the *in vitro* BBB model constructed of hCMEC/D3 cells only was 4.84×10^{-3} cm/min, which was in the same order of magnitude to that (1.65×10^{-3} cm/min) reported by Weksler *et al.* (5). TEER measurements took place, too.

Indeed, hCMEC/D3 cells formed tight junctions as it was seen on a TEM microscope (Fig. 1). The tight junctions are demonstrated as highly dark dense areas between the membranes of the adjacent endothelial cells (indicated with the arrows in Fig. 1). This image is very similar to the one reported by Ceccheli (20) and Underwood *et al.* (21). The gap between the cells seen before and after that area is due to dehydration/fixation protocol used in this experiment, which causes the cells to retract and loose their contact. Also, the expression of zonula occludens by the particular cells was another indication of BBB characteristic (Fig. 2). The arrows in Fig. 2 indicate the punctuated fluorescence located on the membranes of the adjacent endothelial cells. Similar images were reported by Weksler (5) and Ceccheli (20).

Paracelular permeability of all models was assessed using fluorescently labeled dextran of various molecular weights (4 kDa, 40 kDa, 70 kDa) (Table I). Dextrans 40 and 70 kDa have hydrodynamic diameters of 4.8 and 6.5 nm, respectively (22). It was obvious that as the molecular weight of dextran increased, the permeability through the BBB model decreased.

Interestingly enough, it seems that the permeability of the BBB increased slightly in presence of DAOY cells which is the fact in *in vivo* conditions (Table I). However, VC312R cells did not alter the BBB model permeability.

On day 7 of culture, *TEER* measurements were conducted for all *in vitro* models established, such as hCMEC/D3 monolayer, hCMEC/D3-DAOY co-culture, and hCMEC/D3-VC312R co-culture. The average values obtained were of 2, 2, and 6 Ω cm^2 respectively.

Transferrin Receptor Expression on hCMEC/D3 Cells

Anti-Transferrin receptor antibody, anti-CD71, recognizes the human Tfr extracellular domain (23), and is capable of

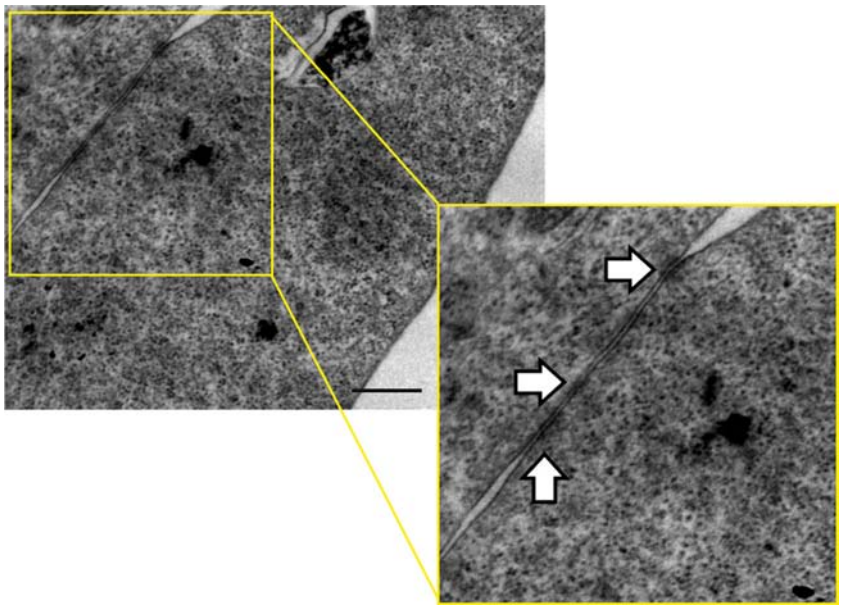

Fig. 1 Characterization of BBB consisted of hCMEC/D3 cells using TEM. The tight junctions due to the presence of occludin are illustrated as highly dark dense areas on the cell membranes between the adjacent endothelial cells. (The gap between the cells seen before and after that area is due to dehydration/fixation protocol used in this experiment, which causes the cells to retract and loose their contact). [Magnification 26,500×,].

binding to receptor molecules on the plasma membrane. Therefore, hCMEC/D3 cells were incubated with anti-Transferrin receptor primary antibody at 4°C, and then Alexa Fluor 488 was used for the fluorescence staining as a secondary antibody. Human Tfr was found to be expressed on the hCMEC/D3 plasma membrane as shown by the immunostaining assay (Fig. 3).

Transferrin receptor expression was investigated on the surface of both DAOY and VC312R cells because one of the hypotheses was that after transcytosis of immunoliposomes, they will be taken up at a higher extent by the tumor cells due to the overexpression of Tfr (24).

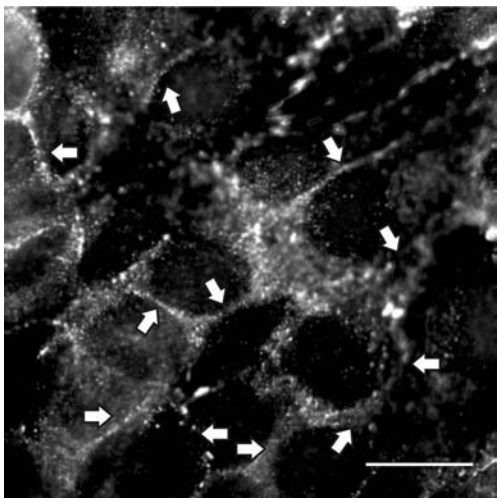

Fig. 2 Characterization of BBB consisted of hCMEC/D3 cells using anti-occludin antibodies. The *arrows* in Fig. 2 indicate the punctuated fluorescence located on the membranes of the adjacent endothelial cells. [Magnification 100×, bar 20 μm].

Interestingly, DAOY cells expressed Tfr on their surface, while VC312Rs did not (data not shown).

Liposome Preparation and Characterization

Liposomes were made using the lipid film method (according to this method, small unilamellar vesicles were produced as it has been reported by Torchilin and Weissig) (25). They were either fluorescently or radioactively labeled according to the experimental purpose. Their physicochemical characteristics are presented in Table II. Liposomes size was approximately 130 nm and zeta potential close to 0 indicating an adequate coating of the particle surface by PEG molecules. When anti-Tfr antibody was attached at the end of the PEG moieties, the particle size increased as expected but it increased significantly more than anticipated. This might have been caused due to homopolymerization of antibody molecules during the activation procedure of the carboxyl-groups of the protein. Antibodies carry amine and carboxyl groups on their surface and maybe some homopolymerization might have been encouraged during the carboxyl-group activation period. Even then, the zeta-potential was low enough (−0.5 mV) which showed that the particles were still coated with PEG molecules. The antibody was attached to the amino-group at the end of the PEG moieties *via* an amide bond. Coupling reaction yield was 85% as it was obtained using Bradford assay. The final antibody/phospholipid ratio was 118 μg of antibody/mg of lipid. Also, antibody retention on the liposome surface was evaluated using [^{125}I]-labeled antibody in both PBS and cell growth medium (EGM-2) enriched with 15% of fetal bovine serum at 37°C (Fig. 1S, suppl. material). Immuno-ARLs were stable in the conditions used, since approximately 80% of

Table I Permeability of fluorescently-labelled dextran of various molecular weights through the *In Vitro* bbb and bbb-tumour co-culture models

FITC-Dextrans (kDa)	Permeability coefficients ($\times 10^{-3}$ cm/min) [sd]		
	hCMEC/D3	hCMEC/D3 + DAOY	hCMEC/D3 + VC312R
4	0.741 [0.05]	0.917 [0.06]	0.555 [0.11]
40	0.0637 [0.001]	0.0746 [0.009]	0.0609 [0.001]
70	0.0446 [0.006]	0.0528 [0.001]	0.0413 [0.002]

antibody was retained on the liposome surface after 24 hs of incubation in both media tested (Fig. 1S, suppl. material).

Also, anti-Tfr-ARLs were relatively stable after 24 hs of incubation in 80% serum as they exhibited 70% of calcein retention. Calcein release was used as an estimate of the immune-ARL stability (Fig. 2S, suppl material).

Despite the unexpected larger liposome size, anti-Tfr-ARLs were taken up by endothelial cells in a specific way as it was shown by the complete uptake inhibition after cell pre-incubation with transferrin. Indeed, immunoliposome uptake occured by hCMEC/D3 cells, a phenomenon completely inhibited after pre-incubation of cells with free trasnferrin which is a natural endogenous compound binding specifically to the transferrin receptor (Fig. 3S, suppl. material).

Effect of Arsonoliposomes on Endothelial Cells

Cytotoxicity Studies

The specific toxicity of the ARLs on tumor cells has been very well established (13,14). However, the toxic effect that ARLs might have on the hCMEC/D3 cells was investigated by the MTT assay and early apoptosis assays. Figure 4 shows the effect of PEG-conventional and PEG-ARLs on the endothelial cell line incubated with a range of concentrations from 200 to 1,000 μg/mL for a period of 1–4 days. Figure 4a shows that the conventional liposomes did not affect the cells. The outcome was the same after the cell incubation with the arsonoliposomes. IC_{50} was not reached even after 4 days of incubation with 1,000 μg/mL of PEG-ARLs where cell viability was approximately 60%. Liposome concentration 400 μg/mL was considered safe enough for 1 day incubation time.

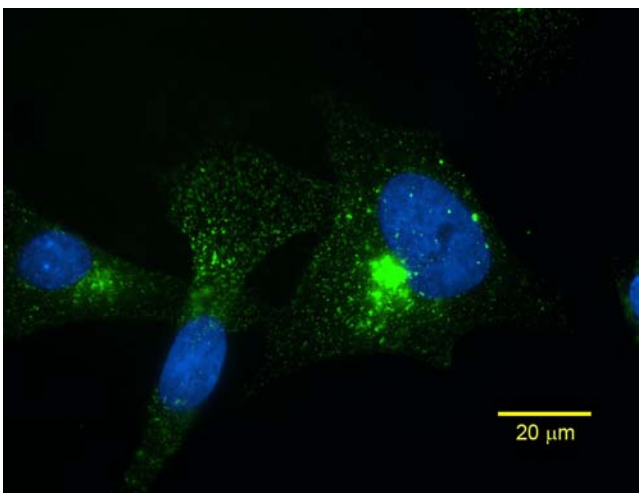

Fig. 3 Immunofluorescence staining of hCMEC/D3 cells for Transferrin receptor expression. Cell monolayer grown on coverslips was incubated with anti-CD71 primary antibody (Abcam) and then with Alexa Fluor 488 (*green*) as secondary antibody. Transferrin receptor is located on the cell surface. Cell nuclei were stained with DAPI in *blue*.

Early Apoptosis Studies

In order to cross reference findings from the MTT assay and conclude to an acceptable time to set up the experimental protocol using the *in vitro* BBB models, early apoptosis studies were carried out using the Annexin V method. Figure 5 shows early apoptosis due to Annexin V which is fluorescently labeled with Alexa Fluor 488 (green), while necrosis is indicated in red due to propidium iodide permeation into the nucleus. Cells were incubated with positive and negative controls for apoptosis such as TNF-α and PEG-DSPC/Chol liposomes (400 μg/mL), respectively (Fig. 5). Cells incubated with PEG-ARLs showed signs of early apoptosis after 5 hs of incubation and after 24 hs of incubation had been undergone necrosis. Therefore, based on the MTT and the early apoptosis studies, incubation of cells with 400 μg/mL for 4 hs was chosen as experimental set up to investigate the permeability of liposomes through the *in vitro* BBB and BBB-tumor co-culture models.

Table II Physicochemical properties of liposome formulations

Formulation	Size [sd] (nm)	ζ-potential [sd] (mV)
ARLs		
PEG-ARLs	131 [3.58]	−1.2 [0.21]
Bodipy PEG-ARLs	126 [2.17]	−0.9 [0.03]
[^{3}H]-Anti-Tfr-ARLs	412.6 [4.1]	−0.5 [0.09]
DSPC/Chol (used as control)		
PEG-DSPC/Chol	127 [5.24]	−0.6 [0.34]

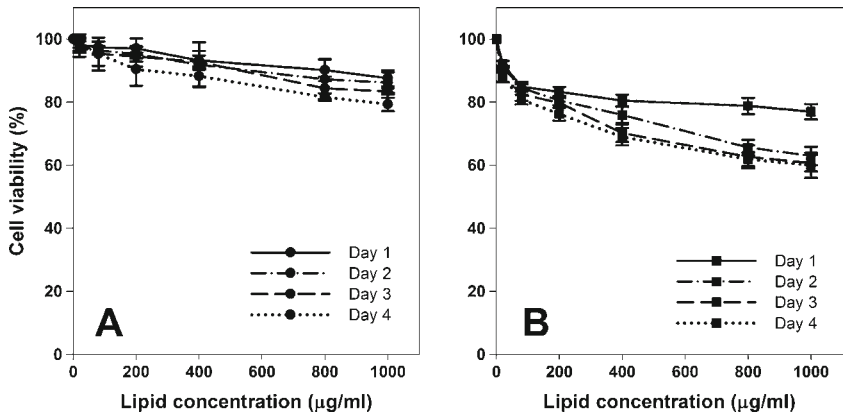

Fig. 4 Cytotoxicity effect of PEG-conventional (**a**) and PEG-ARLs (**b**) on hCMEC/D3 cells. Cells were incubated with a range of liposome concentrations for a period of 1–4 days. Each concentration for each day was tested in triplicates and all experiments were performed in triplicates.

Permeability of PEG-ARLs and Anti-Tfr-ARLs Through the *In-Vitro* BBB and BBB-Tumor Models

Permeability of the liposomal formulations in presence and in absence of the antibody was performed using liposomes containing tritiated cholesterol (Table III). It is obvious that the anti-Tfr-ARLs apparent permeability was much higher to that of plain PEG-ARLs in either the *in vitro* BBB or the BBB-tumor cell co-culture models. There was an 8-fold increase of permeability of immunoliposomes through the *in vitro* BBB model alone and that of the co-culture with DAOY cells in comparison to non-specific PEG-ARLs. However, there was a 6-fold increase through the co-culture with the VC312R cells. A possible explanation for that difference is probably the effect of VC312R cells on the endothelial cells because they seem to contribute to a tighter BBB, which is somewhat unexpected considering the facts in *in vivo* conditions. Therefore, it would be interesting to investigate in more detail the hCMEC/D3 – VC312R and the hCMEC/D3 – DAOY communication and any changes that this might cause.

Moreover, the ratio of anti-Tfr-ARLs permeability through the various *in vitro* BBB models in absence and in presence of the medulloblastoma cells was 1.86 and 1.57 for VC312R and DAOY cells, respectively.

Moreover, anti-Tfr-ARL uptake by the DAOY aggregates in the 3D model was 2.6-fold higher (DAOY: ~3,700 DPM/mg/5×10^5 cells, VCR: ~1,400 DPM/mg/5×10^5 cells) than that by the VC312R aggregates in the relevant 3D model ($p<0.01$, Student's *t*-test). This effect can be probably attributed to the expression of transferrin receptors by DAOYs only (VC312Rs do not express Tfr) that led to an increased retention of the antibody bearing arsonoliposomes.

To confirm that the radioactive signal was attributed to liposomes and not to free ^{3}H-cholesterol, 20 μl of the medium in the abluminal compartment were withdrawn, diluted with PBS and analyzed for size. Particles of 140 and 420 nm in diameter were detected which confirmed the presence of PEG-ARLs and anti-Tfr-ARLs, respectively.

Additionally, in order to exclude the effect of the size difference between immunoarsonoliposomes and plain arsonoliposomes, PEGylated multilamellar vesicles (MLVs) of similar size (386 nm, [ζ-potential: −1.6 mV]) to immunoarsonoliposomes were produced. The apparent permeability of the PEGylated-MLVs through the blood brain barrier model consisted of hCMEC/D3 cells, was only 1.23×10^{-7} cm/s in comparison to 7.56×10^{-7} cm/s shown by anti-Tfr-ARLs.

These results indicate that the immunoarsonoliposome permeability was not affected by the size but it was a receptor specific phenomenon.

DISCUSSION

The current work was an attempt to establish a 3D *in vitro* co-culture model of tumor-BBB to address the needs for better understanding of drug and nanoparticle transport into the brain as well as to provide an alternative method to animal testing according to replacement-reduction-refinement principles. To our knowledge, this is the first *in vitro* blood brain barrier-tumor model comprising endothelial and cancer (medulloblastoma) cell lines in the form of spheroids.

Morphology of the blood brain barrier model consisting of only endothelial cells was initially observed. The presence of tight junctions between hCMEC/D3 cells was revealed by TEM studies (Fig. 1), while the expression of ZO-1 protein was highlighted by immunocytochemistry studies (Fig. 2). The presence of ZO-1 protein is a strong indication of formation of tight junctions because ZO-1 is a cytoplasmic protein contributing to the restriction of paracellular permeability by being attached to one end of occludin, a transmembrane protein which, also, contributes to tight junction formation (26). The expression of transferrin receptor on the endothelial cells was also confirmed (Fig. 3). It was attempted, for the first time, to co-culture this model with three-dimensional brain tumor

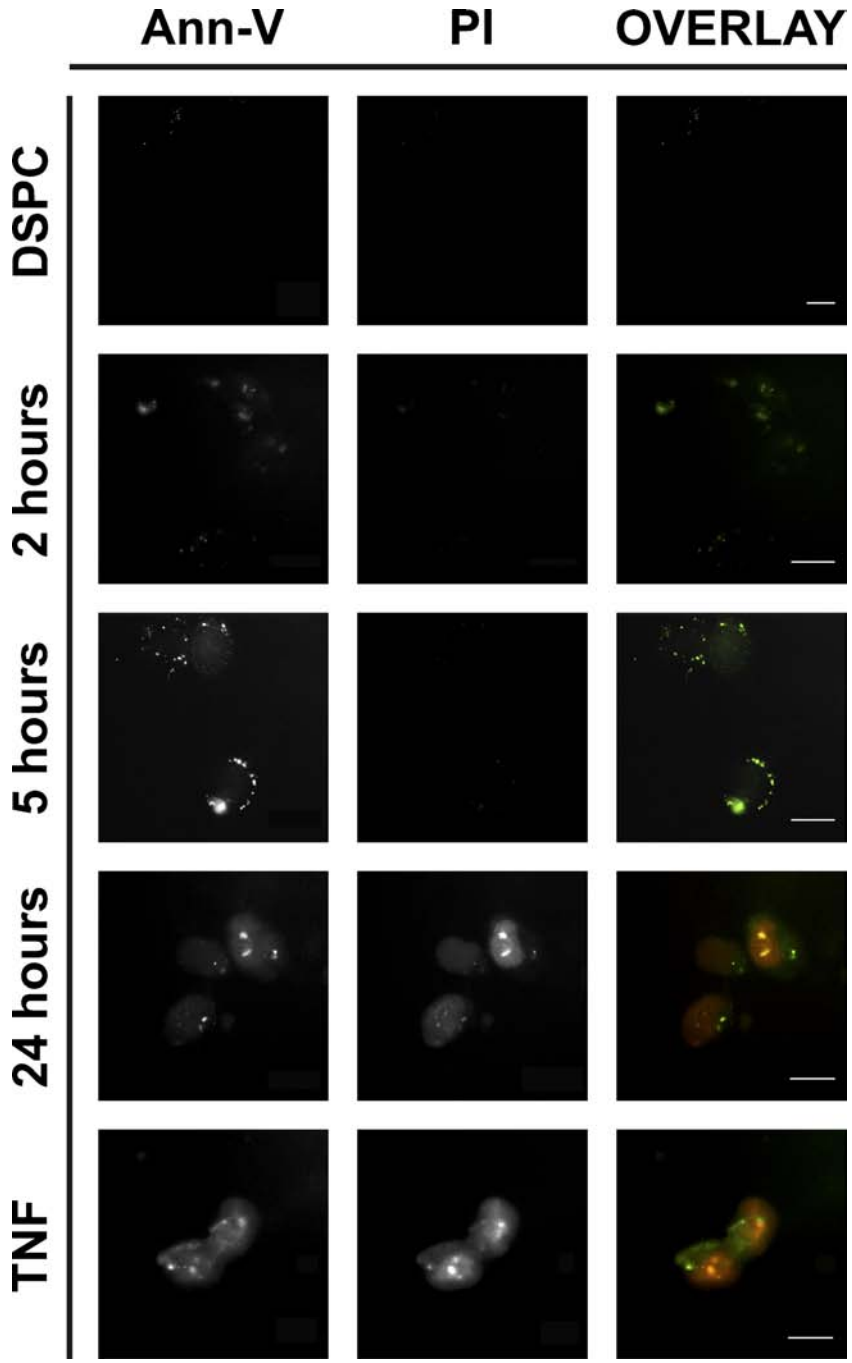

Fig. 5 Fluorescence microscope images of hCMEC/D3 cells after incubation with TNF-α, 200 μg/ml of arsonoliposomes for 2 h, 5 h, 24 h and 200 μg/ml of DSPC liposomes for 5 h. TNF-α (50 ng/ml) was added as positive control for apoptosis induction and PEG-DSPC/Chol liposomes at 200 $\mu g/10^6$ cells were used as negative control for apoptosis induction. Dead cell nuclei were stained red by PI, early apoptotic cells were stained green (Annexin V). Bar: 10 μm.

Table III [^{3}H]-ARLs and [^{3}H]-anti-Tfr-ARLs apparent permeability through the *In Vitro* BBB and BBB-Tumour Co-culture models

Type of *in vitro* model	Apparent permeability (10^{-7} cm/s) [sd]	
	PEG-ARLs	Anti-Tfr-ARLs
hCMEC/D3	0.87 [0.012]	7.56 [0.36]
hCMEC/D3 + VC321R	2.48 [0.19]	14.1 [1.61]
hCMEC/D3 + DAOY	1.45 [0.16]	11.9 [1.20]

models seeding spheroids of medulloblastoma (either VCR or DAOY) on the abluminal side of the Transwell apparatus while endothelial cells were growing as monolayer on the luminal side.

The paracellular permeability of all models established was tested using fluorescently labelled dextrans of different molecular weights in the range of 4 to 70 kDa as well as TEER measurements. Moreover, paracellular permeability of the *in vitro* model consisted of hCMEC/D3 cells alone was tested using [^{14}C]-sucrose. Radioactive sucrose permeability was low

enough (4.84×10^{-3} cm/min) and comparable to that reported for the same cell line (5.53×10^{-3} cm/min – fluorescein with M.W. 382 was used as a marker) (27). However, Weksler *et al.*, reported a value of 1.89×10^{-3} cm/min (5). Sucrose permeability for another immortalized primary human brain capillary endothelial cells (BB19) was much higher (11.7×10^{-3} cm/min) (28). Also, rat brain endothelial cell lines such as RBE4 and GPNT showed permeability values of 12.85×10^{-3} cm/min (sucrose-M.W. 342 used as marker) and 7.08×10^{-3} cm/min (fluorescein-M.W. 332 used as a marker), respectively (29,30). However, the model is not as tight as that formed by brain capillary endothelial cells in co-culture with astrocytes (0.75×10^{-3} cm/min) (20). Therefore, given all data available (those produced by this study and those available in the literature), the *in vitro* BBB model developed here in absence of medulloblastoma spheroids is characterized by quite low values of permeability to sucrose which indicates a quite tight barrier.

Additionally, TEER measurements of all models showed very low values as it was mentioned in the result section. The same *in vitro* BBB (hCMEC/D3) model showed TEER values between 40 and 70 Ω cm^2 (5,27). TEER measurements of *in vitro* BBB models made of immortalized primary human brain capillary endothelial cells have shown low TEER values; *e.g.* NKIM-6 showed 100 Ω cm^2 (4). On the other hand, bovine BMEC monolayers have shown much higher values (500 Ω cm^2) (31). Primary rat and bovine brain endothelial cell monolayers have shown 8 and 4.1 Ω cm^2 (32,33). It is obvious that there is a great range of TEER values generated by the various *in vitro* BBB models developed and it depends on the type of the cell type used. Therefore, combination of hCMEC/D3 with astrocytes and pericytes or in presence of other important factors should be explored further.

Permeability of dextran of various molecular weights was found to follow the same trend as it was reported elsewhere (5,27).

In this work we studied the permeability of liposomes through the barrier. The liposomes we tested had a size of approximately 120 nm (small unilamellar vesicles) and 400 nm (multilamellar vesicles). Permeability of dextran through the barrier either alone or in co-culture with the cancer cell spheroids was quite low (appr. 0.06 for 40 kDa dextran with hydrodynamic diameter of 4.8 nm and appr. 0.05 for 70 kDa dextran with hydrodynamic diameter of 6.5 nm). Therefore, the expected paracellular permeability for studying the liposome permeability through the *in vitro* models would be expected to be very low or zero. Moreover, it was clear that the presence of the tumors influenced the development and the tightness of the barrier, but the effects were different depending on the medulloblastoma cell line (Table I). DAOYs increased the BBB permeability as expected (34) while VCRs showed the opposite effect. This unexpected effect by VCRs can be possibly attributed to the heterogeneity of the primary tumors that the two medulloblastoma cell lines were derived from. Another hypothesis is that, in the process of immortalization one cell type usually dominates: this may be of neural or glial origin, but also a totally differentiated tumor type. It has been demonstrated (35) that glia has a deep influence in modulating the permeability of endothelial cells. In particular, the factors secreted by astroglia and microglia in basal conditions contribute to decrease the blood brain barrier permeability. But, in activated conditions, microglia and astrocytes can release soluble factors, such as TNF-α that contribute to permeabilization of the blood brain barrier. Therefore, the more glia-like the cell is, the more likely it is to induce changes in the tight junctions.

One of the main aims of this work was to study the permeability of arsonoliposomes through the *in vitro* blood brain barrier models to investigate whether the latter can be delivered specifically to the brain using the transferrin receptor. Permeability studies were carried out by adding radiolabelled plain and immunoarsonoliposomes into the abluminal side of the models. Incubation time was limited to 5 h, as previous experiments have shown that by prolonging the contact of arsonoliposomes with endothelial cells would cause apoptosis with a consequent loss of blood brain barrier characteristics (14). It was evident that plain arsonoliposomes crossed the *in vitro* blood brain barrier models but the effect was significant for the co-culture models of the BBB with the tumor aggregates. In fact, the apparent permeability was 2-fold higher in presence of the tumor aggregates (Table III).

When using arsonoliposomes directed to the transferrin receptor, the apparent permeability was increased at least 5-fold in comparison to plain arsonoliposomes, suggesting that transcytosis can occur *via* the transferrin receptor (Table III). Therefore, active targeting was successfully achieved, as immunoarsonoliposomes can cross the barrier in a significantly higher extent than plain arsonoliposomes, despite their larger size. Non-specific PEGylated-MLVs of the same size as the immunoarsonoliposomes exhibited 6-fold drop in the apparent permeability through the BBB model consisted of the hCMEC/D3 cells only. It is also worth to highlight that the presence of tumors increased the permeability of arsonoliposomes significantly, regardless the functionalization. This suggests that arsonoliposomes would likely enter the brain in presence of neoplastic diseases, while they won't be able to pass the barrier in physiological conditions, unless they are carrying the Anti-Tfr-antibody.

CONCLUSIONS

The three-dimensional models between the BBB and the tumor aggregates were successfully established; they showed the basic *in-vivo* characteristics of the barrier, and as such might be appropriate models to study the transport of various

drugs and nanoparticles through the healthy and diseased BBB. The latter was well proven by the use of plain arsonoliposomes and immunoarsonoliposomes.

Those nanocarriers were transferred effectively through the BBB model to the tumor aggregate *via* the transferrin receptor. This also showed the potential of the particular liposomes as an anticancer therapy due to good tolerance of the *in vitro* BBB model to the toxic effect of the nanocarriers.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors are grateful to the Medway School of Pharmacy, University of Kent for funding the research project as a PhD studentship. The authors would like to thank Dr. Ignacio Romero (The Open University, Milton Keynes) for providing the hCMEC/D3 cell line and Prof. Geoff Pilkington (UoPortsmouth) for providing the VC312R cell line.

Abdulghani Al-Shehri and Marco E. Favretto contributed equally to the research work.

REFERENCES

1. Pardridge WM. Drug targeting to the brain. Pharm Res. 2007;24(9):1733–44.
2. Cucullo L, Aumayr B, Rapp E, Janigro D. Drug delivery and *in vitro* models of the blood-brain barrier. Curr Opin Drug Discov Devel. 2005;8(1):89–99.
3. Prudhomme JG, Sherman IW, Land KM, Moses AV, Stenglein S, Nelson JA. Studies of Plasmodium falciparum cytoadherence using immortalized human brain capillary endothelial cells. Int J Parasitol. 1996;26(6):647–55.
4. Ketabi-Kiyanvash N, Herold-Mende C, Kashfi F, Caldeira S, Tommasino M, Haefeli WE, *et al.* NKIM-6, a new immortalized human brain capillary endothelial cell line with conserved endothelial characteristics. Cell Tissue Res. 2007;328(1):19–29.
5. Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, *et al.* Blood-brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J. 2005;19(13):1872–4.
6. Poller B, Gutmann H, Krahenbuhl S, Weksler B, Romero I, Couraud PO, *et al.* The human brain endothelial cell line hCMEC/D3 as a human blood-brain barrier model for drug transport studies. J Neurochem. 2008;107(5):1358–68.
7. Cucullo L, Couraud PO, Weksler B, Romero IA, Hossain M, Rapp E, *et al.* Immortalized human brain endothelial cells and flow-based vascular modeling: a marriage of convenience for rational neurovascular studies. J Cereb Blood Flow Metab. 2008;28(2):312–28.
8. Boado RJ, Pardridge WM. The Trojan horse liposome technology for nonviral gene transfer across the blood-brain barrier. J Drug Deliv. 2011;2011:296151.
9. Huynh GH, Deen DF, Szoka FC. Barriers to carrier mediated drug and gene delivery to brain tumors. J Control Release. 2006;110(2):236–59.
10. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J Control Release. 2000;65(1–2):271–84.
11. Evens AM, Tallman MS, Gartenhaus RB. The potential of arsenic trioxide in the treatment of malignant disease: past, present, and future. Leuk Res. 2004;28(9):891–900.
12. Kallinteri P, Fatouros D, Klepetsanis P, Antimisiaris SG. Arsenic trioxide liposomes: encapsulation efficiency and *in vitro* stability. J Liposome Res. 2004;14(1–2):27–38.
13. Gortzi O, Papadimitriou E, Kontoyannis CG, Antimisiaris SG, Ioannou PV. Arsonoliposomes, a novel class of arsenic-containing liposomes: effect of palmitoyl-arsonoli pid-containing liposomes on the viability of cancer and normal cells in culture. Pharm Res. 2002;19(1):79–86.
14. Favretto ME, Marouf S, Ioannou P, Antimisiaris SG, Parker TL, Kallinteri P. Arsonoliposomes for the potential treatment of medulloblastoma. Pharm Res. 2009;26(10):2237–46.
15. Cecchelli R, Berezowski V, Lundquist S, Culot M, Renftel M, Dehouck MP, *et al.* Modelling of the blood-brain barrier in drug discovery and development. Nat Rev Drug Discov. 2007;6(8):650–61.
16. Grabarek Z, Gergely J. Zero-length crosslinking procedure with the use of active esters. Anal Biochem. 1990;185:131–5.
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
18. Stewart JC. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem. 1980;104(1):10–4.
19. Koda Y, Shiotani K, Toth I, Tsuda Y, Okada Y, Blanchfield JT. Comparison of the *in vitro* apparent permeability and stability of opioid mimetic compounds with that of the native peptide. Bioorg Med Chem Lett. 2007;17(7):2043–6.
20. Cecchelli R, Dehouck B, Descamps L, Fenarta L, Buée-Scherrera V, Duhem C, *et al.* In vitro model for evaluating drug transport across the blood–brain barrier. Adv Drug Deliv Rev. 1999;36:165–78.
21. Underwood JL, Murphy CG, Chen J, Franse-Carman L, Wood I, Epstein DL, *et al.* Glucocorticoids regulate transendothelial fluid flow resistance and formation of intercellular junctions. Am J Physiol. 1999;277(2 Pt 1):C330–42.
22. Choi JJ, Wang S, Tung YS, Morrison 3rd B, Konofagou EE. Molecules of various pharmacologically-relevant sizes can cross the ultrasound-induced blood-brain barrier opening in vivo. Ultrasound Med Biol. 2010;36:58–67.
23. Ortiz-Zapater E, Soriano-Ortega E, Marcote MJ, Ortiz-Masiá D, Aniento F. Trafficking of the human transferrin receptor in plant cells: effects of tyrphostin A23 and brefeldin A. Plant J. 2006;48(5):757–70.
24. Wen DY, Hall WA, Conrad J, Godal A, Flørenes VA, Fodstad O. *In vitro* and *in vivo* variation in transferrin receptor expression on a human medulloblastoma cell line. Neurosurgery. 1995;36(6):1158–63. discussion 1163-4.
25. Torchilin V, Weissig, V. Liposomes 2nd ed., A practical approach. Oxford University Press; 2003.
26. Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J Cell Biol. 1994;127:1617–26.
27. Förster C, Burek M, Romero IA, Weksler B, Couraud PO, Drenckhahn D. Differential effects of hydrocortisone and TNFα on tight junction proteins in an in vitro model of the human blood–brain barrier. J Physiol. 2008;586:1937–49.
28. Kusch-Poddar M, Drewe J, Fux I, Gutmann H. Evaluation of the immortalized human brain capillary endothelial cell line BB19 as a human cell culture model for the blood–brain barrier. Brain Res. 2005;1064:21–31.
29. Rist RJ, Romero IA, Chan MWK, Couraud PO, Roux F, Abbott NJ. F-Actin cytoskeleton and sucrose permeability of immortalised rat brain microvascular endothelial cell monolayers: effects of cyclic AMP and astrocytic factors. Brain Res. 1997;768:10–8.
30. Regina A, Romero IA, Greenwood J, Adamson P, Bourre JM, Couraud PO, *et al.* Dexamethasone regulation of P-glycoprotein

activity in an immortalized rat brain endothelial cell line, GPNT. J Neurochem. 1999;73:1954–63.
31. Deri MA, Abraham CS, Kataoka Y, Niwa M. Permeability studies on in vitro blood–brain barrier models: physiology, pathology, and pharmacology. Cell Mol Neurobiol. 2005;25:59–127.
32. Hayashi K, Nakao S, Nakaoke R, Nakagawa S, Kitagawa N, Niwa M. Effects of hypoxia on endothelial/pericytic co-culture model of the blood–brain barrier. Regul Pept. 2004;123:77–83.
33. Furie MB, Naprstek BL, Silverstein SC. Migration of neutrophils across monolayers of cultured microvascular endothelial cells. An in vitro model of leucocyte extravasation. J Cell Sci. 1987;88:161–75.
34. Mayhan WG. VEGF increases permeability of the blood-brain barrier via a nitric oxide synthase/cGMP dependent pathway. Am J Physiol. 1999;276(5 Pt 1):C1148–53.
35. Prat A, Biernacki K, Wosik K, Antel JP. Glial cell influence on the human blood-brain barrier. Glia. 2001;36(2):145–55.