British Journal of Pharmacology (2003) 140, 1201–1210
www.nature.com/bjp

Alkylglycerol opening of the blood–brain barrier to small and large fluorescence markers in normal and C6 glioma-bearing rats and isolated rat brain capillaries

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1 The blood–brain barrier (BBB) represents the major impediment to successful delivery of therapeutic agents to target tissue within the central nervous system. Intracarotid alkylglycerols have been shown to increase the transfer of chemotherapeutics across the BBB.

2 We investigated the spatial distribution of intracarotid fluorescein sodium and intravenous lissamine-rhodamine B200 (RB 200)–albumin in the brain of normal and C6 glioma-bearing rats after intracarotid co-administration of 1-*O*-pentylglycerol (200 mM). To elucidate the mechanisms involved in the alkylglycerol-mediated BBB opening, intraluminal accumulation of fluorescein isothiocyanate (FITC)–dextran 40,000 was studied in freshly isolated rat brain capillaries using confocal microscopy during incubation with different alkylglycerols. Furthermore, 1-*O*-pentylglycerol-induced increase in delivery of methotrexate (MTX) to the brain was evaluated in nude mice.

3 Microscopic evaluation showed a marked 1-*O*-pentylglycerol-induced extravasation of fluorescein and RB 200–albumin in the ipsilateral normal brain. In glioma-bearing rats, increased tissue fluorescence was found in both tumor tissue and brain surrounding tumor. Confocal microscopy revealed a time- and concentration-dependent accumulation of FITC–dextran 40,000 within the lumina of isolated rat brain capillaries during incubation with 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol, indicating enhanced paracellular transfer *via* tight junctions. Intracarotid co-administration of MTX and 1-*O*-pentylglycerol (200 mM) in nude mice resulted in a significant increase in MTX concentrations in the ipsilateral brain as compared to controls without 1-*O*-pentylglycerol ($P<0.005$).

4 In conclusion, 1-*O*-pentylglycerol increases delivery of small and large compounds to normal brain and brain tumors and this effect is mediated at least in part by enhanced permeability of tight junctions.

British Journal of Pharmacology (2003) **140,** 1201–1210. doi:10.1038/sj.bjp.0705554

Keywords: Alkylglycerol; blood–brain barrier; brain tumor; drug delivery; fluorescence markers; methotrexate; rat brain capillaries; tight junction; confocal microscopy

Abbreviations: BBB, blood–brain barrier; BSA, bovine serum albumin; BW, body weight; FITC, fluorescein isothiocyanate; FPIA, fluorescence polarization immunoassay; MTX, methotrexate; RB 200, lissamine-rhodamine B200

Introduction

The brain capillary endothelium plays a key role in the pathophysiology of various diseases of the CNS such as inflammatory disorders, tumors, ischemia, and seizures (Pardridge, 1998). The mechanisms of functional regulation and transport at the blood–brain barrier (BBB) are not well understood, and further investigation is needed to clarify both how BBB dysfunction is mediated in disease states and how transport systems at the barrier can be altered for therapeutic purposes. Currently, successful treatment of many brain disorders seems to be impossible even though highly active drugs exerting powerful effects at the target site have been developed. Their efficacy is limited by very low penetration across the BBB (Neuwelt *et al*., 1999). Thus, enhancing or targeting drug delivery to the brain has become a major issue in experimental neurology. Chemical modification of drugs and the use of transport mediating agents or vector systems are possible strategies for drug targeting to the brain (Smith, 1993; Huwyler *et al*., 1996; Reszka *et al*., 1997; Kroll & Neuwelt, 1998; Jolliet-Riant & Tillement, 1999); however, the clinical benefit of such measures has still to be demonstrated. In contrast to this, methods to open the BBB such as hyperosmotic disruption of the BBB or bradykinin receptor-induced increase in barrier permeability have already been shown to be effective in the treatment of experimental brain tumors (Blasberg *et al*., 1990; Nomura *et al*., 1994; Elliott *et al*., 1996; Matsukado *et al*., 1996; Kroll *et al*., 1998). Chemo-

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Advance online publication: 3 November 2003

therapy of malignant brain tumors in conjunction with osmotic opening of the BBB has been advanced to the stage of clinical trials and tumor response has been documented in both adults and pediatric patients (Gumerlock *et al*., 1992; Dahlborg *et al*., 1996; 1998; Doolittle *et al*., 2000).

The inadequate drug delivery across the BBB is a major factor that explains the poor response rates of chemosensitive brain tumors (Siegal & Zylber-Katz, 2002). Consequently, there is a need for new maneuvers designed to overcome the limited access of anticancer agents to the brain and to brain tumors. Recently, the transfer of a variety of chemotherapeutic drugs across the BBB was shown to be increased dramatically by intracarotid drug administration in the presence of short-chain alkylglycerols (Erdlenbruch *et al*., 2000; 2002). The intracarotid injection of alkylglycerols resulted in a concentration-dependent accumulation of the coinjected drugs within the brain. The effect was rapidly reversible and variations in the chemical structure of the alkylglycerols allowed for modulation of the extent of increased barrier permeability (Erdlenbruch *et al*., 2003). Of particular importance is the fact that there were no signs of toxicity in long-term experiments using intracarotid 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol in rats (Erdlenbruch *et al*., 2003). In view of their low toxicity and the potent and well-controllable effects, intracarotid alkylglycerols are thought to be a very promising principle to facilitate the transport of therapeutics across the BBB. However, little is known about the distribution of the delivered drugs within the hemispheres and whether large compounds such as proteins will enter the brain after intracarotid alkylglycerols. Furthermore, there are only marginal insights into the mechanisms involved in alkylglycerol-mediated BBB opening. Finally, nude mice represent the species mostly used for the treatment of experimental human brain tumor xenografts, but alkylglycerols were not administered in nude mice so far.

Therefore, the purpose of the present study was (a) to estimate the 1-*O*-pentylglycerol-mediated increase in the penetration of small and large fluorescence markers into the brain of both normal and glioma-bearing rats and to investigate the spatial distribution of the different markers within the brain, (b) to elucidate the mechanisms of action of the alkylglycerols by studying the accumulation of fluorescein isothiocyanate (FITC)–dextran 40,000 (40 kDa) in freshly isolated rat brain capillaries using confocal microscopy and quantitative image analysis during incubation with different alkylglycerols, and (c) to demonstrate feasibility and effectivity of the permeabilizing effect of 1-*O*-pentylglycerol in nude mice by intracarotid co-administration with methotrexate (MTX).

Methods

Drugs

The synthesis of 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol has been described in detail elsewhere (Erdlenbruch *et al*., 2000). Purity of the products was assessed by HPLC and was above 99% in all experiments. Depending on the binding site of the alkyl group, up to 1% of the respective 1-*O*- or 2-*O*-positional alkyl isomers were found. No other by-products were identified. For the *in vivo* experiments, 1-*O*-pentylglycerol was used as the prototypical compound of the alkylglycerols and because long-term *in vivo* studies have demonstrated a lack of toxicity (Erdlenbruch *et al*., 2003). Incubation with rat capillary preparations was performed *in vitro* using 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol, an effective derivative of the alkyldiglycerol family associated with no toxic effects at therapeutic levels (Erdlenbruch *et al*., 2003).

Fluorescence markers of different molecular size were chosen to estimate the permeability of the BBB in the presence or absence of 1-*O*-pentylglycerol. In the animal experiments, low and high molecular fluorescence dyes were administered. Fluorescein sodium (MW 367 Da) was purchased from Merck (Darmstadt, Germany), and lissamine-rhodamine B200 (RB 200) and human albumin were obtained from Sigma-Aldrich (Deisenhofen, Germany). Fluorescein sodium was dissolved in physiological saline, forming a stock solution of 5%. For intracarotid use (80 mg kg^{-1}), this solution was further diluted to a final concentration of 2.5% in accordance with the protocol for the respective treatment groups. RB 200 was coupled to albumin and purified as described by Klein *et al*. (1986). FITC–dextran 40,000 (MW 40 kDa, Sigma-Aldrich) was used for *in vitro* investigation of alkylglycerol-dependent permeation of drugs across isolated rat brain microvessels.

MTX was purchased from Onco-Hexal AG (Holzkirchen, Germany) and a dose of 5 mg kg^{-1} body weight (BW) was given to nude mice. Control animals received intra-arterial MTX without 1-*O*-pentylglycerol. In these experiments, MTX was diluted with physiological saline to the desired final volume (100 μl). For intracarotid coinjection with 1-*O*-pentylglycerol, a mixture of alkylglycerol and MTX was diluted with water. The concentration of 1-*O*-pentylglycerol in the final solution was 200 mM and the osmolality of the injected solutions ranged from 421 to 448 mosm kg^{-1}. Within this range of osmolality, no permeability changes due to osmotic effects have to be expected.

Animal experiments

Male Wistar rats and male nude mice were kept under conventional controlled conditions (22°C, 55% humidity, and day–night rhythm) and had free access to a standard diet (V1534-000, Fa. sniff, Soest, Germany) and tap water. The nude mice were purchased from Charles River Laboratories (CD®-1 nude mice, Crl:CD1®-*nu*, Sulzfeld, Germany). The experiments were carried out in accordance with the German Law on the Protection of Animals.

The differential permeability of the BBB in the absence and presence of 200 mM 1-*O*-pentylglycerol was investigated in tumor-free rats ($n = 19$) and in rats bearing C6 gliomas ($n = 12$) using small and large fluorescence markers. The delivery of MTX to the brain of tumor-free nude mice was evaluated in the absence and presence of 1-*O*-pentylglycerol ($n = 12$). MTX was chosen for these experiments because (a) it is used in different treatment protocols of pediatric brain tumors, (b) it is known to exhibit poor penetration into the CNS after both intravenous and intra-arterial administration (Neuwelt *et al*., 1998; Erdlenbruch *et al*., 2000), and (c) the effect of alkylglycerols on the transfer of MTX into the CNS has been thoroughly investigated in rats (Erdlenbruch *et al*., 2003).

Tumor implantation

Wistar rats weighing 180–220 g received intraperitoneal ketamine/xylazine (90 μg/7.5 μg per g BW) and 1×10^5 C6

cells were inoculated into the right putamen as described previously (Erdlenbruch *et al*., 1998). Briefly, rats were placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, U.S.A.) and 10 μl of a suspension of C6 cells in RMPI 1640 medium was injected using a 10-μl Hamilton syringe with a 26-gauge needle 1 mm anterior and 3 mm lateral to the bregma, and 5 mm deep to the dural surface. Tumors were allowed to grow until first signs of manifestation (first day of weight loss or second day of no weight gain, 14 ± 2 days after tumor implantation). At this time, large tumors were found with only minor variability in tumor size (Figure 4).

Intra-arterial drug administration

The intracarotid administration of the drugs to rats was performed as described previously (Erdlenbruch *et al*., 2003). In brief, rats were anesthetized with intraperitoneal pentobarbital (50 mg kg^{-1} BW) followed by intravenous injections. Body temperature was maintained at 37.5°C, and arterial blood pressure and heart rate were monitored by a Statham transducer (Gould, Oxnard, CA, U.S.A.) throughout the experiments *via* the left femoral artery. The right external carotid artery was cannulated and the microcatheter system was filled with blood in a retrograde manner. Fluorescein sodium (80 mg kg^{-1} BW) or MTX (5 mg kg^{-1} BW) was injected into the right internal carotid artery either in the presence (simultaneously) or in the absence of 1-*O*-pentylglycerol (200 mM, mean dose 90 ± 10 mg kg^{-1} BW). All solutions were heated to 37°C and sterile filtered immediately before administration. In rats, a total volume of 1.2 ml consisting of 800 μl drug solution followed by rinsing with 400 μl of isotonic saline was injected with a flow rate of 6 ml min^{-1} using a Hamilton dispenser (Microlab, Hamilton Bonaduz, Switzerland). During the injection, the common carotid artery was clamped. RB 200–albumin (200 μl per 100 g BW) was administered intravenously 3 min before the intracarotid bolus of fluorescein sodium. RB 200–albumin was given to stain intravascular space as well as to assess the permeabilizing effect of 1-*O*-pentylglycerol on large compounds. A simultaneous intracarotid co-injection of both fluorescent dyes was not performed, since the viscosity and the volume of the infusate would be too high. At 5 min after the intracarotid administration of fluorescein, the brains were rapidly removed and frozen in isopentane (−50°C).

The nude mice were anesthetized by intraperitoneal ketamine/xylazine (75 mg per kg BW/5 mg per kg BW). The surgical procedure was the same as described for the rat experiments; however, the trachea and the femoral artery were not cannulated. A special fine and bent glass catheter was used to cannulate the external carotid artery. MTX (5 mg kg^{-1} BW) was administered simultaneously or without 1-*O*-pentylglycerol (200 mM, 105 ± 29 mg kg^{-1} BW). A volume of 100 μl of drug solution was injected followed by rinsing with 40 μl of isotonic saline (total volume 140 μl). At 5 min after the MTX administration, mice were perfused with Ringer's solution *via* the left ventricle, the brains were rapidly removed and stored at −20°C until further analysis.

Histological evaluation

For histology, the frozen brains were sectioned into 7 μm slices. Either sagittal or coronal serial sections were made and air dried. Evaluation of the sections was performed using fluorescence microscopy (Zeiss Universal, Zeiss Göttingen, Germany). Na-fluorescein and RB 200 were visualized by use of filter combinations as described by Klein *et al*. (1986). The histological preparations were assigned to the respective anatomical planes according to the stereotaxic atlas from König & Klippel (1963). Serial coronal planes were used for quantitative interindividual comparison of the fluorescence intensity of the brain. The reference plane (zero) for coronal sections was a plane through the interaural line and distances were given from this plane to identify the respective section levels (König & Klippel, 1963). Fluorescence intensity was measured semiquantitatively using a high-resolution black and white camera (Kappa, CF8/1 DXC, Gleichen, Germany) and computerized image analysis.

Analysis of MTX concentrations

The concentrations of MTX in the brain tissue were determined separately in the right hemisphere (ipsilateral to the injection), in the left hemisphere (contralateral), and in the cerebellum (including brain stem) as described previously (Erdlenbruch *et al*., 2000). In brief, organs were minced and homogenized in alkaline medium (NaOH 0.1 M, total volume 0.8 ml, pH = 12–13). After neutralization with hydrochloric acid, MTX concentrations were determined by fluorescence polarization immunoassay (FPIA; Jolley *et al*., 1981). The FPIA reagent systems were purchased from Abbott Laboratories, IL, U.S.A., and analyses were performed according to the operation manuals. Calibration curves for tissue concentrations of MTX were established for each assay. Values are given as pmol mg^{-1} wet weight.

In vitro *experiments*

Capillary isolation Rat brain capillaries were isolated as described by Miller *et al*. (2000). In brief, capillaries (3–6 animals per preparation) were isolated using a modification of the procedure of Pardridge *et al*. (1985). All steps were carried out at 4° C in pre-gassed (95% O_2/5% CO_2) solutions. Keeping the tissue on ice and in well-gassed buffers was essential for preservation of transport function. Pieces of grey matter were gently homogenized in three volumes (v w^{-1}) of buffer A (103 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM HEPES) and, after addition of dextran (final concentration 30%), the homogenate was centrifuged at low speed. The resulting pellet was resuspended in buffer B (buffer A supplemented with 25 mM $NaHCO_3$, 10 mM glucose, 1 mM Na-pyruvate, and 0.5% (w/v) bovine serum albumin (BSA)) and then filtered through a 200 μm nylon mesh. The filtrate was passed over a glass bead column and, after washing with 500 ml buffer, the capillaries adhering to the beads were collected by gentle agitation. Capillaries were centrifuged, the pellet resuspended in ice-cold, gassed, BSA-free Krebs–Henseleit buffer and immediately used for transport experiments.

Confocal microscopy

Confocal microscopy was performed as described (Miller *et al*., 2000). Briefly, capillaries were transferred to a covered Teflon incubation chamber containing 1.5 ml of pre-gassed

Krebs–Henseleit medium with 1 μM FITC–dextran 40,000 in the absence or presence of 1-*O*-pentylglycerol or 2-*O*-hexyldiglycerol at concentrations up to 20 mM. The chamber floor was a 4 × 4 cm glass coverslip to which the capillaries adhered and through which they could be viewed. All experiments were conducted at room temperature (18–20°C). The chamber was mounted on the stage of a Zeiss LSM 5 Pascal inverted confocal laser scanning microscope and viewed through a ×63 or ×40 water immersion objective. The 488-nm laser line, a 510-nm dichroic filter, and a 515-nm long-pass emission filter were employed. Low laser intensity was used to avoid photobleaching of the dyes. With the photomultiplier gain set, tissue autofluorescence was undetectable. Capillaries were first viewed under reduced transmitted light illumination. A field containing 2–5 capillaries was selected and a confocal fluorescence image was obtained. Capillaries contained mostly unbranched segments at least 100 μm in length and were 5–8 μm in diameter at pixel resolutions 0.2 μm pixel^{-1}. Transmitted light and fluorescence micrographs showed the endothelium to be 1–1.5 μm thick.

Fluorescence intensities were measured from stored images using NIH Image 1.61 or Scion Image software as described previously (Miller, 1995). Owing to microscope adjustments and focusing the capillaries selected, the first image could be made 4 min after starting incubation. Since the appearance of FITC–dextran 40,000 within the capillary lumen was mediated by passive diffusion, equilibrium has to be expected at maximum effects. The background fluorescence intensity was subtracted and the average pixel intensity for each area was calculated. The value assigned to a capillary was the means of all selected areas. As there are uncertainties in relating cellular fluorescence to the actual concentration of an accumulated compound in cells and tissues with complex geometry (Sullivan *et al*., 1990; Miller & Pritchard, 1991), data are reported here as average measured pixel intensity rather than estimated dye concentration.

Statistical evaluation

Mean values ± s.d. are presented unless otherwise indicated. For statistical analyses, one-way analysis of variance (ANOVA) was used.

Results

In vivo *experiments*

To investigate the spatial distribution of small and large fluorescence markers in normal tumor-free rats, serial sections of the brains were analyzed at defined planes. In the absence of 1-*O*-pentylglycerol (control animals, $n = 5$), fluorescein sodium and RB 200–albumin were detected only within the lumina of the cerebral vasculature and no differences in fluorescence intensity were registered between the ipsilateral and the contralateral hemisphere (Table 1). Intracarotid injection of 1-*O*-pentylglycerol resulted in a marked transfer of both fluorescein and RB 200–albumin into the brain tissue ipsilateral to the injection ($n = 14$, Figures 1 and 2). Almost no extravasation of the fluorescent dyes was found in the contralateral hemisphere. The ratio of ipsilateral to contralateral fluorescence intensity in the coronal sections was chosen to quantify the 1-*O*-pentylglycerol-induced increase in BBB permeability. The mean ratio for fluorescein amounted to 6.45 ± 1.4 and for RB 200–albumin to 2.66 ± 1.0 (Table 1). The increase in the fluorescence intensity of fluorescein was considerably stronger than of the intravenously administered high molecular RB 200–albumin. Since the two fluorescent probes were not administered at the same site and circulation times were different, the fluorescence intensities could not be compared. Intraindividual regional differences in the extent of ipsilateral dye extravasation became apparent by analyzing serial sections from the entire brain (Figure 3). Contralateral tissue fluorescence was low in all brain areas (Figure 3). As visualized in Figures 1 and 2, extravasation of the fluorescence markers was slightly more prominent in the cortical regions. Only small interindividual variations were found when comparing the pattern of the 1-*O*-pentylglycerol-induced increase in fluorescence intensity of both of the fluorescent markers (Table 1).

In contrast to the brain of tumor-free rats, basal vascular permeability of intracerebral C6 tumors was increased as demonstrated by intratumoral extravasation of the fluorescent markers in the absence of 1-*O*-pentylglycerol (Figure 4a). The intracarotid co-injection with 1-*O*-pentylglycerol resulted in a conspicuous additional transfer of fluorescein sodium and RB

Table 1 Ratio of fluorescence intensity ipsilateral to contralateral hemisphere after intravenous RB 200–albumin and intracarotid fluorescein sodium in the presence or absence of intracarotid 1-*O*-pentylglycerol

Treatment group	*Level of section*	*Ratio of fluorescence intensity ipsilateral/contralateral*	
		Na-fluorescein	*RB 200–albumin*
Without pentylglycerol (controls, $n = 5$)	Mean of all sections	1.06 ± 0.2*	0.95 ± 0.1*
With 1-*O*-pentylglycerol 200 mM ($n = 7$)	Mean of all sections	6.45 ± 1.4*	2.66 ± 1.0*
	A 11050 μm	5.55 ± 2.0**	2.16 ± 0.7**
	A 9650 μm	7.32 ± 2.0**	2.03 ± 0.2**
	A 6790 μm	5.68 ± 0.7**	2.18 ± 0.7**
	A 4230 μm	6.43 ± 0.8**	3.01 ± 1.4**
	A 2180 μm	6.83 ± 1.9**	3.41 ± 0.1**

The intensity of fluorescence was determined separately in the ipsilateral and contralateral hemisphere using serial coronal sections. The ipsi- to contralateral ratio of fluorescence intensity is depicted. To compare sections from different animals, planes were assigned according to a stereotaxic atlas (König & Klippel, 1963). Distances of the section planes anterior (A) to the auditory canal were used for further evaluation. Values given are means ± s.d.; *$P < 0.05$, means without *versus* means with 1-*O*-pentylglycerol; **$P < 0.05$, fluorescence intensities at defined section planes without 1-*O*-pentylglycerol *versus* with 1-*O*-pentylglycerol; one-way analysis of variance (ANOVA).

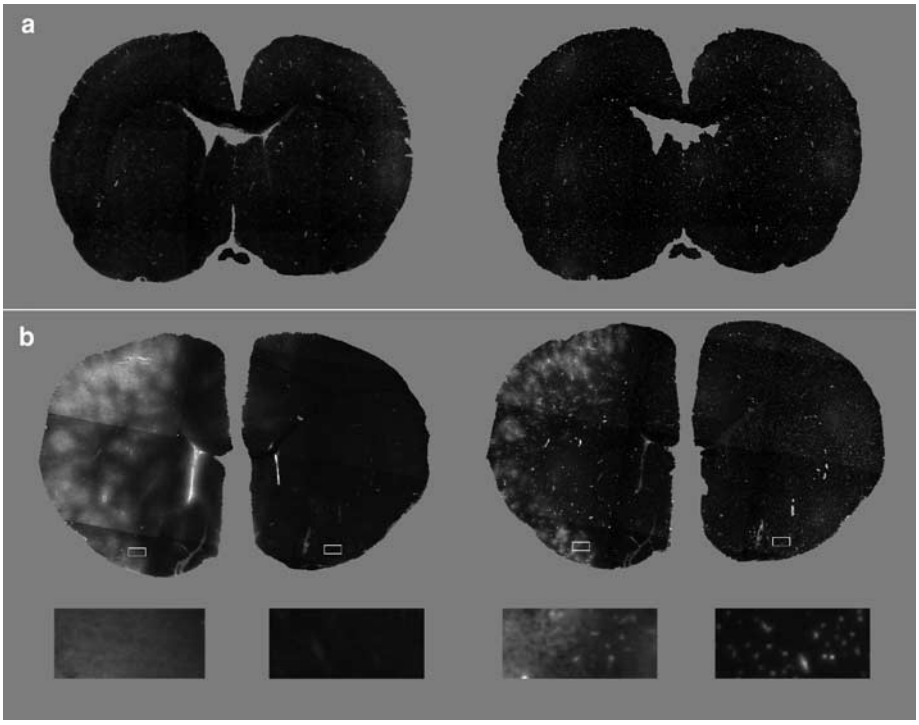

Figure 1 Spatial distribution of tissue fluorescence of small and large fluorescence markers (fluorescein sodium and RB 200–albumin) in normal rat brain in the absence (a) or presence (b) of intracarotid 1-*O*-pentylglycerol (200 mM): coronal sections. Serial coronal sections of frozen brains were obtained and observed by fluorescence microscopy (left panel: FITC; right panel: RB 200–albumin). Fluorescence strictly restricted to the brain vasculature (a) and 1-*O*-pentylglycerol-induced increase in tissue fluorescence of the ipsilateral hemisphere (b). Boxes: Magnification of the respective brain region. Representative sections from one of seven experiments.

200–albumin to both tumor tissue and surrounding ipsilateral brain (Figure 4b). Owing to the excessive increase in fluorescence intensity within the tumors, overexposure of slices showing parts of the tumor was observed even if exposure times were substantially reduced. In view of the high brightness in tumor tissue after 1-*O*-pentylglycerol, variations in the conditions for the computerized imaging and the analysis of fluorescence intensity (e.g. exposure times and additional filters) were necessary to obtain usable results. Therefore, no statistical evaluation of tissue staining was feasible in the tumor experiments.

In an additional series of experiments, intracarotid MTX was given in the presence or absence of 200 mM 1-*O*-pentylglycerol to tumor-free nude mice. After intracarotid administration of MTX without alkylglycerols, low tissue concentrations were found in the brain parenchyma. MTX delivery to the brain was markedly increased by 1-*O*-pentylglycerol. The increase of MTX concentrations was found predominantly in the right hemisphere ipsilateral to the bolus injection ($P<0.05$; Figure 5).

In vitro *experiments*

The permeation of FITC–dextran 40,000 across the walls of freshly isolated rat brain capillaries was measured using confocal laser scanning microscopy. Figure 6 shows representative experiments using control and 1-*O*-pentylglycerol-exposed capillaries. In the control capillary, little change in fluorescence was observed after 20 min. In contrast, 1-*O*-pentylglycerol-exposed capillaries showed rapid increases in fluorescence. We used quantitative image analysis to measure dextran permeation into capillary lumens. Figure 7a shows little change in luminal fluorescence of control capillaries even after 2 h incubation in FITC–dextran-containing buffer. In contrast, capillaries exposed to 2 or 10 mM 1-*O*-pentylglycerol showed a steady increase in luminal fluorescence over 30 min. Addition of 1-*O*-pentylglycerol to control capillaries elicited a rapid and sustained increase in luminal fluorescence (Figure 7b). The effects of both 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol were concentration dependent (Figure 7c), with concentrations as low as 0.2 mM significantly increasing luminal fluorescence after 20 min exposures. From these dose–response data, 1-*O*-pentylglycerol appeared to be slightly more effective. Together, the data indicate that alkylglycerols caused a rapid and concentration-dependent increase in capillary permeability to a marker of paracellular permeation.

Discussion

The limited access of potentially helpful therapeutics into the CNS resulting from the presence of the BBB emphasizes the importance of developing strategies for overcoming the BBB. Only a limited number of approaches to increase the transfer of drugs to the brain have been used in clinical studies so far (Cornford & Hyman, 1999). Osmotic opening of the BBB by intracarotid infusion of hypertonic mannitol solution has been reported to increase the delivery of water-soluble drugs, peptides, antibodies, and viral vectors to the brain (Rapoport, 2000). This technique is used in conjunction with intra-arterial chemotherapy to treat human primary CNS lymphomas or

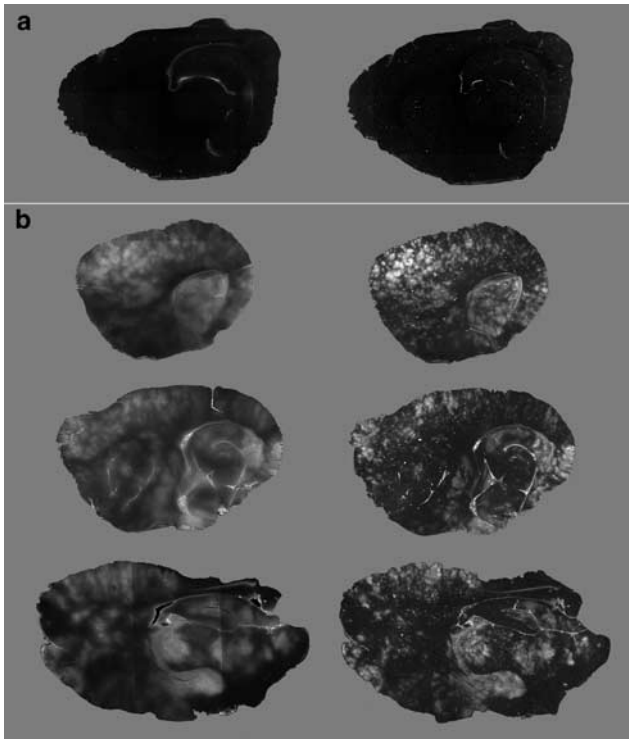

Figure 2 Spatial distribution of tissue fluorescence of small and large fluorescence markers (fluorescein sodium and RB 200–albumin) in normal rat brain in the presence of intracarotid 1-*O*-pentylglycerol (200 mM): sagittal sections. Serial sagittal sections of frozen brains were obtained and observed by fluorescence microscopy (left panel: FITC; right panel: RB 200–albumin). Lack of tissue fluorescence in the contralateral hemisphere (a) and increased fluorescence in the ipsilateral hemisphere after intracarotid 1-*O*-pentylglycerol (b). Representative sections from one of seven experiments.

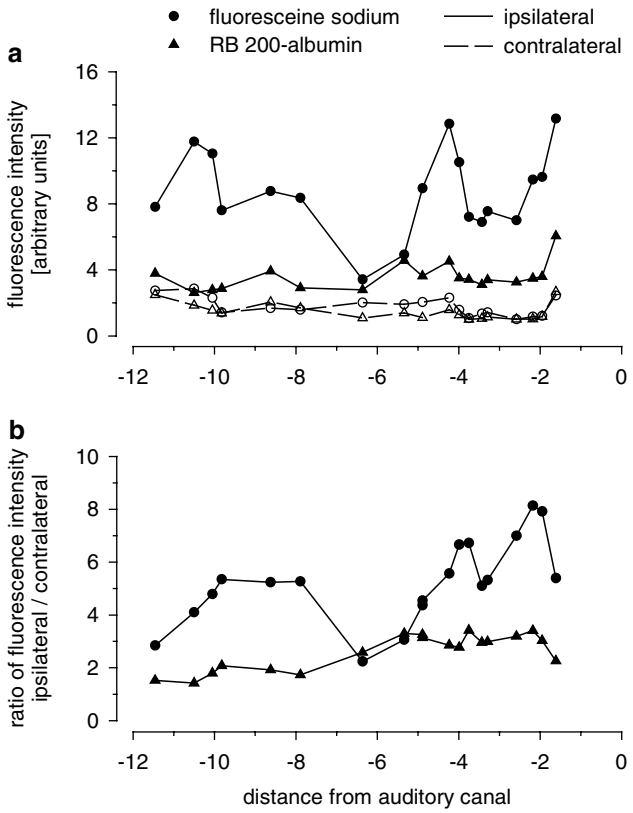

Figure 3 Fluorescence intensity of fluorescein sodium and RB 200–albumin in serial coronal brain sections after co-administration with 1-*O*-pentylglycerol in normal rats. RB 200–albumin was administered intravenously 3 min before an intra-arterial co-injection of 1-*O*-pentylglycerol (200 mM) and fluorescein sodium *via* the internal carotid artery. Serial coronal sections were obtained at defined planes anterior to the interaural plane. Fluorescence intensity was determined separately in the ipsilateral and contralateral hemisphere using a computerized camera system. (a) Fluorescence intensity of fluorescein sodium and RB 200–albumin in the different brain regions. (b) Values depicted represent the ratio of fluorescence intensity ipsilateral to contralateral of fluorescein sodium (circles) and RB 200–albumin (triangles).

high-grade malignant gliomas, and improved survival has been reported (Dahlborg *et al*., 1996; 1998; Doolittle *et al*., 2000; McAllister *et al*., 2000). However, barrier opening has been shown to last for 6–8 h (Siegal *et al*., 2000), and treatment-related toxicity (Roman-Goldstein *et al*., 1991; Gumerlock *et al*., 1992; Williams *et al*., 1995; Siegal & Zylber-Katz, 2002) as well as a number of methodological difficulties, for example, catheter access, optimal flow rate, thrombotic complications, and choice of anesthetic (Gumerlock & Neuwelt, 1990; Mortimer *et al*., 1992; Rapoport, 2000), has prevented the widespread use of this technique so far. The administration of the bradykinin B2 receptor agonist RMP-7 represents a biochemical method to open the BBB in patients with malignant brain tumors (Gregor *et al*., 1999; Emerich *et al*., 2001; Warren *et al*., 2001). RMP-7-mediated barrier permeabilization is almost restricted to the blood–brain tumor barrier (Nomura *et al*., 1994; Matsukado *et al*., 1996) and only a very modest increase in drug transfer to the tumor was achieved in animal models (Kroll *et al*., 1998). In view of these difficulties, other strategies are in demand for the delivery of neuropharmaceuticals across the BBB.

Intracarotid short-chain alkylglycerols have been reported to induce a strong and transient increase in the transport of chemotherapeutic drugs to the ipsilateral hemisphere (Erdlenbruch *et al*., 2003). One goal of the present study was to evaluate the extent and local distribution of alkylglycerol-mediated delivery of large compounds to the brain. Staining of brain parenchyma with albumin-bound RB 200 demonstrated that even proteins could enter the brain when administered in conjunction with 1-*O*-pentylglycerol. Thus, opening of the BBB using alkylglycerols permits brain delivery of drugs with a wide range of molecular size. From preliminary data using intracarotid globulin coupled fluorescence markers, it was inferred that even larger proteins could be transferred to the brain. In earlier experiments using chemotherapeutic drugs of different molecular size, it was shown that the amount of drug delivered to the CNS using 1-*O*-pentylglycerol decreased with increasing size of the co-injected compounds (Erdlenbruch *et al*., 2000). The higher extravascular fluorescence intensities of small marker substances such as fluorescein sodium concur with the fact that 1-*O*-pentylglycerol-mediated enhancement of drug delivery to the brain depends on the molecular size of the injected drug. Furthermore, within the same brain section, tissue staining with fluorescein was more homogenous compared with RB 200–albumin, which exhibited a more spot-like, patchy extravasation predominantly around the vessels. This

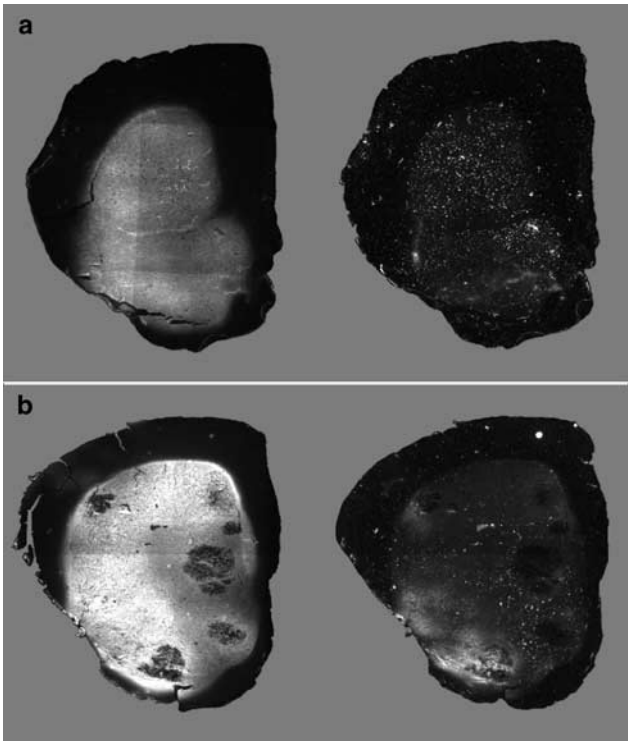

Figure 4 Tissue fluorescence of fluorescein sodium and RB 200–albumin in C6 glioma-bearing rats. Dyes were injected either in the absence (a) or presence (b) of intracarotid 1-*O*-pentylglycerol (200 mM). Dye extravasation in tumors without alkylglycerol treatment (a) and high fluorescence intensity in both tumor and surrounding ipsilateral brain in the presence of 1-*O*-pentylglycerol (b). Note: Four times shorter exposure time in (b) to avoid overexposure of the image. Photographs are representative of six experiments within each group.

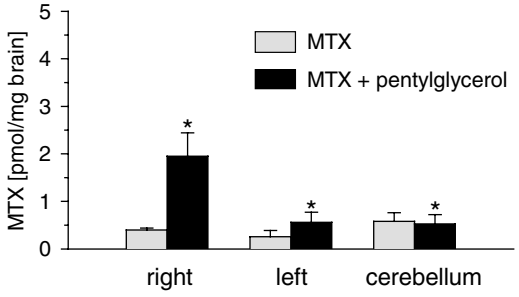

Figure 5 MTX transfer to different regions of the brain of tumor-free nude mice after intracarotid administration of 1-*O*-pentylglycerol. MTX (5 mg kg^{-1}) was given to nude mice ($n=12$) in the absence or presence of 1-*O*-pentylglycerol (200 mM). Right: right hemisphere; left: left hemisphere; cerebellum: cerebellum and brain stem. Concentrations given are means ± s.d.; $*P<0.05$, right *versus* left and right *versus* cerebellum (ANOVA).

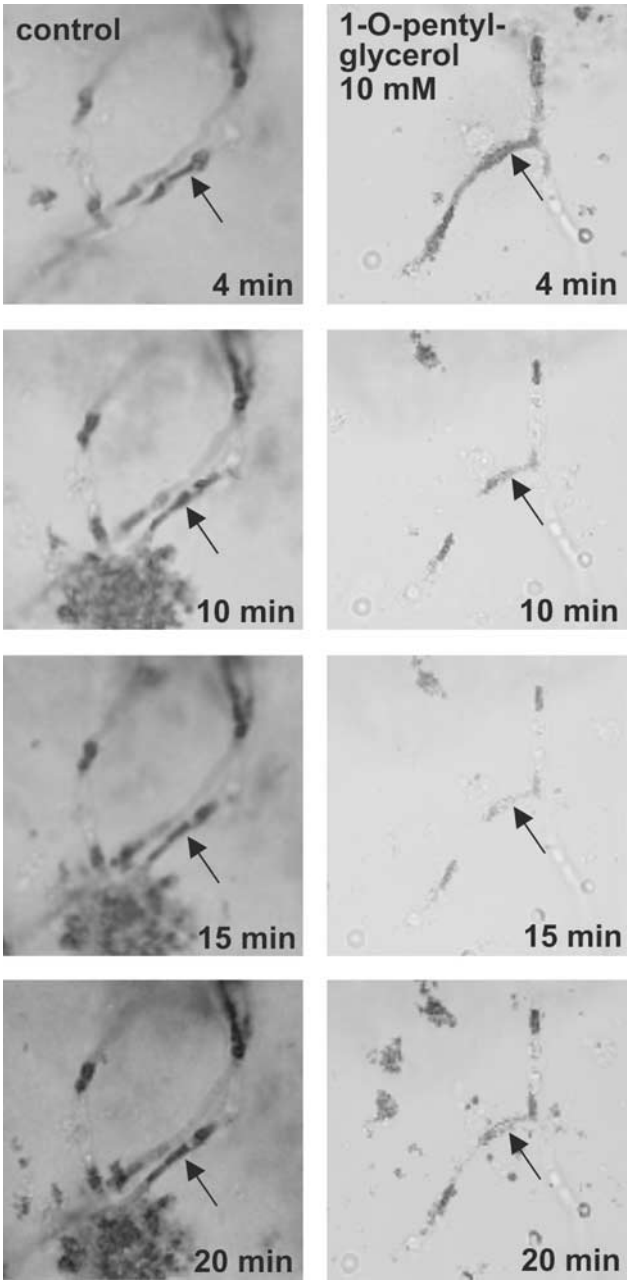

Figure 6 Incubation of freshly isolated rat cerebral capillaries with 1 μM FITC–dextran 40,000 in the absence and presence of 10 mM 1-*O*-pentylglycerol. Owing to microscope adjustments and focusing the capillaries selected, the first image could be made 4 min after starting incubation. Whereas only negligible amounts of the paracellular marker compound in capillary lumens can be seen in control capillaries (left panel), a clear accumulation of FITC–dextran 40,000 was observed within 20 min in the lumina of capillaries incubated with 1-*O*-pentylglycerol (right panels). Arrows indicate the point on which the confocal microscope was focused.

difference in the pattern of fluorescence can be explained by weaker and slower penetration of the large albumin-linked RB 200 into the brain parenchyma. From recent barrier experiments using different chemotherapeutics in rats, it was already assumed that both high molecular size and high polarity of the co-administered drugs are associated with lower CNS penetration, because 1-*O*-pentylglycerol-induced accumulation of vancomycin and gentamicin within the brain was significantly lower than that of cisplatin and MTX (Erdlenbruch *et al*., 2000).

There was little interindividual variation in the regional distribution of the fluorescence markers in normal animals. This provides further evidence of the reliability of barrier opening by 1-*O*-pentylglycerol in the normal unchanged brain. Within the C6 gliomas, baseline permeability was heterogeneous. Increased vascular permeability of brain tumors has

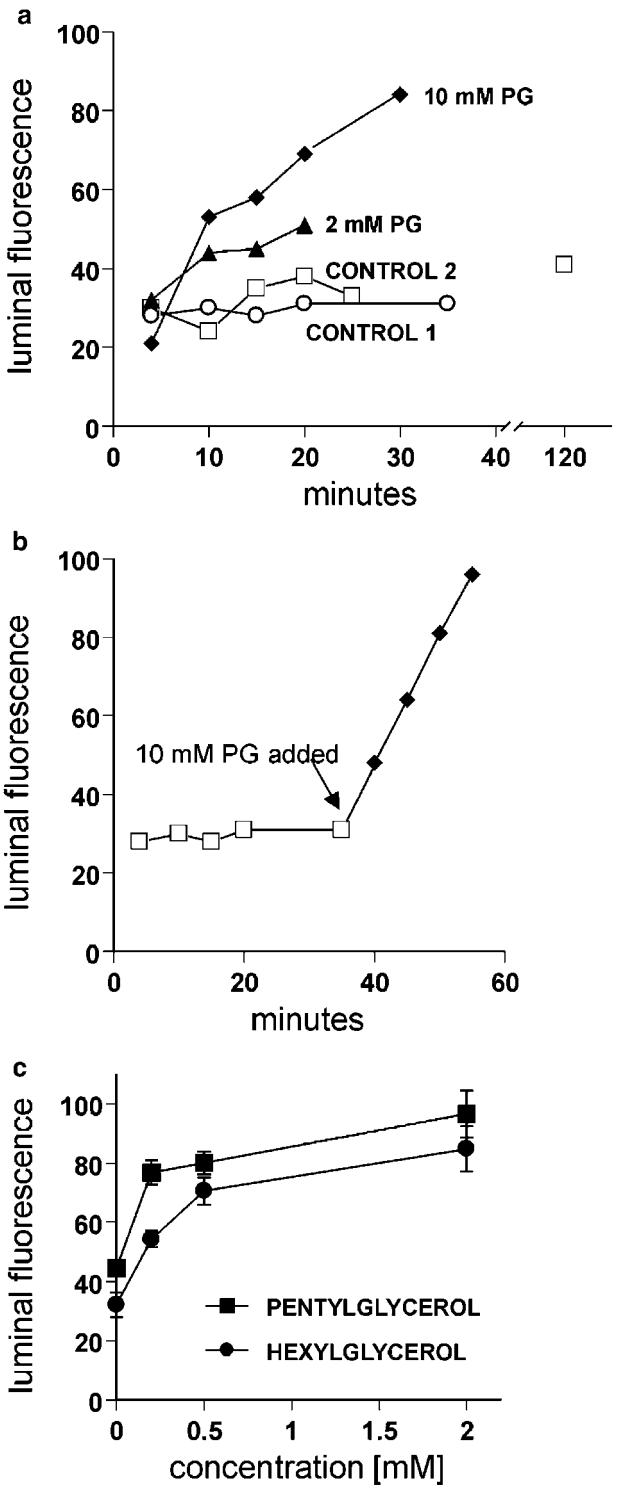

Figure 7 Effect of alkylglycerols on permeation of FITC–dextran 40,000 across the walls of freshly isolated rat brain capillaries. (a) Time course of FITC–dextran 40,000 permeation across individual control and 1-*O*-pentylglycerol-exposed capillaries. (b) Data from a single capillary showing the effect of addition of 10 mM 1-*O*-pentylglycerol. (c) Dose response for 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol. Measurements were made over the first 20 min after transferring capillaries to chambers containing FITC–dextran 40,000 with 0–2 mM alkylglycerol. Data are given as mean fluorescence intensity for 3–6 capillaries; variability is shown as s.d.

been well described in the literature (Hiesiger *et al*., 1986; Inoue *et al*., 1987; Neuwelt *et al*., 1998) and may account for some tumor responses to chemotherapy. In the present study, there was a strong increase in tissue fluorescence in both tumor tissue and surrounding ipsilateral normal brain reflecting similar permeabilizing effects of 1-*O*-pentylglycerol at the blood–brain tumor barrier and the intact barrier. Due to the high fluorescence intensity of the tumor tissue after administration of the fluorescent markers in the presence of 1-*O*-pentylglycerol, the exposure time had to be shortened substantially to avoid overexposure of the images. Therefore, no quantification of the increase in tissue fluorescence within the different brain regions could be performed. In earlier studies, however, the increase in drug delivery to brain tumor tissue and ipsilateral cortex after intracarotid administration of 1-*O*-pentylglycerol was analyzed using different chemotherapeutic drugs (Erdlenbruch *et al*., 2000; 2002). The 1-*O*-pentylglycerol-mediated increase in the transfer of methotrexate to tumor tissue was approximately as high as to the surrounding ipsilateral tumor-free brain (18-fold in the tumor as compared to 28-fold in the surrounding brain). Thus, the use of alkylglycerols in conjunction with intra-arterial chemotherapy enables enhanced access of anticancer drugs to the tumor mass and to infiltrative malignant cells at the tumor edge.

This contrasts with the effects observed after osmotic BBB disruption, because mannitol has been reported to increase drug delivery predominantly to the normal brain rather than to the tumor itself, resulting in a reversal of the tumor-to-cortex permeability relationship (Hiesiger *et al*., 1986; Inoue *et al*., 1987; Shapiro *et al*., 1988; Barnett *et al*., 1995; Neuwelt *et al*., 1998). As the present study allowed no quantification of the 1-*O*-pentylglycerol-mediated increase in tumor uptake of the fluorescent markers, further studies using both mannitol and alkylglycerols are needed to compare the increase in drug transfer to different tumor areas and to the surrounding normal brain. Intra-arterial chemotherapy of brain tumor-bearing animals in conjunction with BBB opening by hyperosmolar mannitol or 1-*O*-pentylglycerol will clarify whether alkylglycerols offer any superiority over mannitol.

Since large molecular weight agents were also transported across the BBB, and in view of both the potential for exact regulation of barrier opening and the lack of long-term toxicity of short-chain alkylglycerol derivatives (Erdlenbruch *et al*., 2003), other applications also appear to be of great promise. Therefore, it is noteworthy that increased drug transfer in the presence of alkylglycerols was easily reproducible in nude mice. The 1-*O*-pentylglycerol-mediated MTX accumulation found in tumor-free nude mice was less marked than in normal Wistar rats (Erdlenbruch *et al*., 2003) indicating that the permeabilizing effect of alkylglycerols may differ between different species. Improved transit of specific brain-targeted compounds to the brain tissue will be of great interest in the next few years because new and effective neuropharmaceutics have been designed recently.

The use of the optical sectioning capabilities of confocal microscopy allowed us to develop a procedure that can provide new insights into the mechanisms of alkylglycerol-associated increase in transendothelial drug transport. The selective intraluminal accumulation of the fluorescent markers indicated that permeation of the drugs was mediated by

enhanced permeability of the zonulae occludens. Relevant transcellular transport could be excluded due to the lack of intracellular labeling of the endothelial cells. Even at high magnification there was no evidence for a transcytotic pathway of the marker. Incubation of low concentrations of alkylglycerols with synthetic membranes consisting of dipalmitoylglycerophosphocholine or dimyristoylglycerophosphocholine resulted in an impressive and concentration-dependent decrease in phase transition temperature (data not shown). From these data, alkylglycerol-induced fluidization of biological membranes was hypothesized, possibly acting *via* changes in tight-junctional integrity. This effect, however, appears to be short-lasting, because baseline permeability of the barrier was restored within a few minutes (Erdlenbruch *et al*., 2000; 2003). Furthermore, no clinical or neuropathological alterations were found 2 and 4 weeks after intracarotid 1-*O*-pentylglycerol treatment (Erdlenbruch *et al*., 2003). Ongoing experiments focusing on functional alterations of tight junction proteins will contribute to further clarify the mechanisms involved in the alkylglycerol-mediated increase in BBB permeability.

In summary, a strong increase in delivery of fluorescence markers of different molecular weight to both normal brain and brain tumors was demonstrated in rats by intracarotid co-administration of 1-*O*-pentylglycerol. Increased drug transfer across the BBB was also observed after intracarotid 1-*O*-pentylglycerol in nude mice. The permeabilizing effect of the alkylglycerols is mediated at least in part by enhanced permeability of the tight junctions.

We thank Ms Thi-Hoang Hoa Nguyen and Ms Regina Krügener for their excellent technical assistance and Dr Lise M. Bjerre for critically reviewing the manuscript. This work was supported by a grant from the Deutsche Krebshilfe (10-1554-Er 2).

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(Received July 14, 2003
Revised September 23, 2003
Accepted September 24, 2003)