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Intracarotid administration of short-chain alkylglycerols for increased delivery of methotrexate to the rat brain

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1 The intracarotid administration of alkylglycerols has been reported previously by us to be a novel strategy for increased delivery of various chemotherapeutic drugs to the normal brain and brain tumors in rats.

2 Effectiveness and structure–activity relations of the most promising pentyl- and hexylglycerol derivatives have been elucidated *in vivo* by analyzing the transfer of methotrexate (MTX) across the blood–brain barrier (BBB) in normal rats. The effects were compared with BBB disruption using hypertonic mannitol or intracarotid infusion of bradykinin. Furthermore, toxicity of the alkylglycerols has been studied in long-term experiments.

3 Apart from 1-*O*-pentyldiglycerol, all alkylglycerols induced a concentration-dependent increase in MTX delivery to the brain varying from 1.1 to more than 300-fold compared to intra-arterial MTX alone. Enhanced barrier permeability rapidly approached baseline values within 5 and 120 min at the latest. Chemical structure, concentration, time schedule of injections and combination of different alkylglycerols were identified as instruments suited to regulate the MTX accumulation within a wide range. Mannitol 1.4 M resulted in very high MTX levels in the brain as observed using the highest concentrations of alkylglycerols. Intracarotid infusion of bradykinin had only a minor effect on the BBB. Using 1-*O*-pentylglycerol or 2-*O*-hexyldiglycerol, both cell culture experiments and long-term *in vivo* analyses including clinical, laboratory and histopathological evaluations revealed no signs of toxicity.

4 In summary, intracarotid short-chain alkylglycerols constitute a very effective and low toxic strategy for transient opening of the BBB to overcome the limited access of cytotoxic drugs to the brain.

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Abbreviations: BBB, blood–brain barrier; BW, body weight; CNS, central nervous system; MTX, methotrexate

Introduction

Malignant brain tumors represent a major therapeutic problem in pediatric oncology because of their high frequency and poor prognosis. In contrast to the favorable results in children suffering from extracranial solid tumors, leukemia or lymphoma, there has been only marginal progress in the chemotherapy of childhood brain tumors (Packer, 1997; Pollack *et al*., 1999; Reddy & Packer, 1999). In addition to the biological heterogeneity of brain tumors resulting in chemoresistance, this is mainly due to the poor penetration of most of available anticancer agents across the blood–brain barrier (BBB) into the brain tissue. Although the barrier may be partially disrupted within the tumor, as evidenced by contrast enhancement on computer tomographic and magnetic resonance imaging scans (Østergaard *et al*., 1999), only a modest increase in vascular permeability has been found in both experimental and human brain tumors (Groothuis *et al*., 1982; Blasberg *et al*., 1990). Furthermore, malignant cells infiltrating the brain immediately adjacent to the tumor or, following migration, distant to the tumor may proliferate behind an intact barrier. As a result, response to chemotherapy is often transient. This emphasizes the need for new strategies in neuro-oncology to circumvent the BBB in order to achieve effective tumor tissue concentrations of chemotherapeutic agents. In the past, only a few methods have been described to increase permeability in normal brain and, most importantly, in brain tumors (Kroll & Neuwelt, 1998). Intracarotid infusions of hypertonic mannitol have been used successfully to open the BBB in animals (Neuwelt *et al*., 1985; Blasberg *et al*., 1990) and in humans (Neuwelt & Rapoport, 1984). In clinical trials, chemotherapy using osmotic BBB disruption was effective in patients with primary lymphoma of the central nervous system (CNS) (Dahlborg *et al*., 1996; 1998; Doolittle *et al*., 2000) as well as in malignant glioma (Gumerlock *et al*., 1992; Kroll & Neuwelt, 1998). However, there is a normal brain *versus* tumor selectivity of osmotic BBB disruption (Hiesinger *et al*., 1986; Inoue *et al*., 1987; Blasberg *et al*., 1990) and significant morbidity has been observed using this

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therapeutic approach (Neuwelt *et al*., 1983; Roman-Goldstein *et al*., 1991). Reproducible and safe osmotic barrier opening in brain tumor chemotherapy seems to be extremely difficult (Siegal *et al*., 2000). Thus, this treatment is still investigational and actually remains restricted to very few centers experienced with this technique. A highly selective increase in the permeability of the brain tumor vasculature has been achieved by the bradykinin analog lobradimil (RMP-7) after both intra-arterial (Inamura & Black, 1994) and intravenous infusions (Elliott *et al*., 1996). A phase I trial using carboplatin and lobradimil in children with refractory brain tumors showed feasibility and tolerable toxicity of this treatment (Warren *et al*., 2001). In animal experiments, however, drug concentrations in brain tumor tissue after RMP-7 have been increased only to a very modest degree (Matsukado *et al*., 1996; Kroll *et al*., 1998).

Recently, we have described a novel and highly effective method for enhanced delivery of drugs to the brain of normal and C6 glioma-bearing rats (Erdlenbruch *et al*., 2000a; 2002). The intracarotid coinjection of short-chain alkylglycerols with different chemotherapeutics resulted in a concentration-dependent enrichment of the coinjected agents predominantly in the ipsilateral hemisphere, whereas the intravenous administration of alkylglycerols has been shown to be ineffective. The alkylglycerol-induced barrier opening was rapidly reversible and the increase in drug concentrations within C6 tumors was as marked as within the surrounding normal brain parenchyma (Erdlenbruch *et al*., 2000a). Of the different alkylglycerols studied, the pentyl- and hexylderivatives seemed to be the most interesting compounds for further evaluation (Erdlenbruch *et al*., 2000b). Since the intracarotid administration of alkylglycerols is considered to be a promising therapeutic principle for brain tumor chemotherapy, this work focuses on effectiveness, regulation, feasibility and toxicity of alkylglycerol-induced opening of the BBB using pentyl and hexyl derivatives. The structure–activity relations of several alkylmono- and alkyldiglycerols to the penetration of methotrexate (MTX) into the brain were investigated in rats and methods to regulate the effect are described. Furthermore, the MTX transfer after alkylglycerol-induced barrier opening was compared with BBB disruption after hypertonic mannitol as well as after pharmacological barrier modification using bradykinin. *In vitro* and long-term *in vivo* experiments demonstrated the lack of toxic side effects and neuropathological changes after intracarotid alkylglycerols.

Methods

Drugs

Alkylglycerols were synthesized as previously described (Erdlenbruch *et al*., 2000a). A pentyl or a hexyl group was bound to glycerol either *via* the primary or the secondary hydroxyl group (Figure 1). The products were purified by silica gel chromatography. Purity was assessed by HPLC and was about 99%. Depending on the binding site of the alkyl group approx. 1% of the respective 1-*O*- or 2-*O*-positional alkyl-isomers were found. No other by-products were observed. Concentrations of the alkylglycerols varied between 10 and 300 mM. To avoid osmotic effects at the BBB, the osmolality of the injected solutions was adapted to iso-osmolar concentrations (mean 310 mosm kg^{-1}). Bradykinin and mannitol were purchased from Sigma-Aldrich (Deisenhofen, Germany). Bradykinin was diluted with physiological saline to a final concentration of 50 mg ml^{-1}. Mannitol was dissolved in water to a concentration of 25% (1.4 M). All solutions were freshly prepared before use, warmed to 37°C and sterile filtered immediately before intracarotid administration.

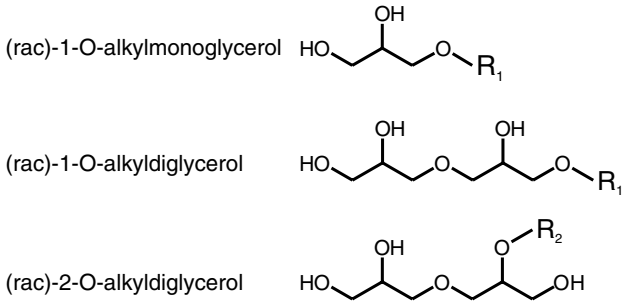

Figure 1 Chemical structures of the alkylglycerols investigated. R_1: pentyl or hexyl; R_2: hexyl.

MTX (Onco-Hexal AG, Holzkirchen, Germany) was used as a model for the transfer of chemotherapeutic drugs into the CNS due to its known poor penetration into the brain after intravenous and intra-arterial administration and its frequent use in both pediatric and adult brain tumor chemotherapy. A dose of 5 mg kg^{-1} body weight (BW) MTX was given. For the intracarotid coinjection with alkylglycerols, a mixture of alkylglycerol and MTX was diluted to the desired alkylglycerol concentration using water and isotonic saline. In control experiments, MTX was dissolved to 800 μl using physiological saline. A total volume of 1.2 ml was injected within 12 s using a Hamilton dispenser (Microlab, Hamilton Bonaduz, Switzerland) with a flow rate of 6 ml min^{-1} consisting of 800 μl drug solution followed by rinsing with 400 μl of isotonic saline.

Cell cultures

To evaluate possible cytotoxic properties of short-chain alkylglycerols *in vitro* human brain tumor cell lines (A 172 and U 87 MG) as well as human monocytes were incubated with 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol. The tumor cell lines A 172 and U 87 MG were purchased from ATCC (Rockville, MD, U.S.A.). Human monocytes were obtained from heparinized blood of healthy donors and prepared as described by Heinemann *et al*. (2000). All tumor cell lines were adapted to RMPI 1640 medium (Boehringer Ingelheim, Germany) supplemented with 10% (v v^{-1}) heat-inactivated (56°C, 30 min) fetal bovine serum (Life Technologies, Karlsruhe, Germany) and 50 μg ml^{-1} gentamycin (Life Technologies, Karlsruhe, Germany). Monocytes were grown in RPMI 1640 medium supplemented with 50 μg ml^{-1} gentamycin and 10% (v v^{-1}) heat-inactivated human serum. Cell lines were grown as monolayers in tissue culture flasks in a humidified atmosphere (5% CO_2/95% air) at 37°C. Drug sensitivity assessment was performed with cells after continuous exposure to 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol for up to 72 h. From pharmacokinetic studies in rats it was known that after intracarotid administration of radiolabeled 1-*O*-pentylglycerol there was a rapid decline in plasma concentrations, and

270 min after the injection the concentrations in plasma and in both hemispheres of the brain ranged between 40 and 60 μM (unpublished data). Therefore, the drug concentrations used to evaluate the toxicity of the alkylglycerols *in vitro* varied from 0.8 to 200 μM. At 24 h after plating, the drugs were added to the cells and the effects on viability and morphology were tested daily. Cytotoxicity of the alkylglycerols was assessed by the mitochondrial cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1.3-benzene disulfonate; WST-1 test, Boehringer Mannheim, Germany) as described elsewhere (Jendrossek *et al*. 1999). Furthermore, morphology of the cells was evaluated daily by polarization light microscopy of native cells as well as by light microscopy of cells fixed in methanol and stained with Wright's solution.

Animal experiments

Male Wistar rats were kept under conventional controlled conditions and had free access to a standard diet (Altromin™) and tap water until the experiment. The experiments have been carried out in accordance with the German Law on the Protection of Animals.

Acute experiments To assess the efficacy of the short-chain alkylglycerols in opening the BBB to chemotherapeutic drugs, the penetration of MTX into the brain of normal tumor-free rats was evaluated *in vivo* ($n=106$ animals). Rats were anesthetized with intraperitoneal pentobarbital (50 mg kg^{-1} BW) followed by intravenous injections, if necessary. Blood pressure and heart rate were recorded via the left femoral artery by a Statham transducer (Gould, Oxnard, CA, U.S.A.). The right external carotid artery was cannulated in a retrograde manner. MTX was injected into the right internal carotid artery in the presence or absence of the different pentyl or hexyl analogs of the alkylglycerols (Figure 1). Antegrade blood flow was interrupted during the intra-arterial injections by clamping the common carotid artery. In an additional experimental group, animals received MTX intravenously without alkylglycerols via the superior vena cava. The volume flow during drug injections was 0.1 ml s^{-1} (800 μl drug solution and 400 μl physiological saline) to avoid flow-induced changes of the drug distribution within the brain vasculature. Thus, animals treated with higher concentrations of alkylglycerols were also given higher doses. To demonstrate that the effect of the alkylglycerols at the BBB is concentration-dependent rather than dose-dependent, further experiments were performed varying either the dose or the concentration of 1-*O*-pentylglycerol. First, different concentrations of 1-*O*-pentylglycerol were administered using a constant dose (118 mg kg^{-1}, $n=9$), and second, animals received different doses of 100 mM 1-*O*-pentylglycerol (46 ± 3 and 118 ± 10 mg kg^{-1}, $n=9$).

The effectiveness of a combined administration of different structural alkylglycerol analogs was also investigated ($n=48$ rats). For this purpose, concentrations of the respective derivatives were used, which exhibit only weak (1-*O*-pentylglycerol 50 and 100 mM, 1-*O*-hexyldiglycerol 50 mM, 2-*O*-hexyldiglycerol 75 mM) or no effects (1-*O*-butylglycerol 100 mM; Erdlenbruch *et al*., 2000a) at the BBB when administered solely with MTX. For the injection of drug combinations, a mixture of two of the alkylglycerols and MTX was freshly prepared (800 μl) and given as described above. The osmolality of the injected solutions varied between 311 and 428 mosm kg^{-1}.

Furthermore, MTX and alkylglycerols were given separately to study the duration of the increased permeability of the BBB. MTX was administered at various times after the injection of the alkylglycerols. Increasing intervals between the injection of alkylglycerol and MTX were studied (3, 15, 30, 60 and 120 min; $n=60$), and compared with both the simultaneous injection and the administration of MTX without alkylglycerol.

In an additional set of experiments ($n=33$ rats), alkylglycerol-induced MTX accumulation was compared with MTX concentrations obtained after osmotic BBB disruption by hypertonic mannitol, and after pharmacological barrier opening by the vasoactive peptide bradykinin. After cannulation of the right external carotid artery, intracarotid 1-*O*-pentylglycerol (120 mM, 320 mosm kg^{-1}) or 2-*O*-hexyldiglycerol (75 mM, 290 mosm kg^{-1}) was injected as described above followed by a continuous infusion of MTX (5 mg kg^{-1}), which was started immediately after the alkylglycerol injection and given for 15 min (53 μl min^{-1}, 290 mosm kg^{-1}). 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol were chosen, because both were used for the toxicological evaluation and 120 mM 1-*O*-pentylglycerol has already been used in other experiments to investigate its pharmacokinetic parameters. A second group of animals was treated by mannitol (1.4 M, 1400 mosm kg^{-1}) given for 30 s at a rate of 0.083 ml s^{-1} (2.5 ml) followed by the continuous intracarotid MTX infusion. Alternatively, bradykinin was infused into the right internal carotid artery (300 mosm kg^{-1}) at a rate of 10 mg kg^{-1} min^{-1} for 15 min. At 5 min after the start of the intracarotid bradykinin infusion, the MTX infusion was started through the same catheter using a Y adapter and maintained for 15 min. Control animals received only intracarotid MTX. In all experiments, 5 min after the MTX administration, blood samples were withdrawn, the left ventricle was cannulated and organs were rinsed with Ringer's solution and rapidly removed. Hematological and serum parameters (see below) were determined in order to evaluate acute toxicity of the alkylglycerols.

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 previously described (Erdlenbruch *et al*., 2000a). Values are given as pmol mg^{-1} wet weight. In brief, organs were minced and homogenized in alkaline medium (NaOH 0.1 M, total volume 2 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 with each assay.

Long-term toxicity experiments In order to estimate long-term adverse effects of the intracarotid administration of alkylglycerols, 18 rats were treated with intra-arterial 1-*O*-pentylglycerol ($n=6$), 2-*O*-hexyldiglycerol ($n=6$) or physiological saline (controls, $n=6$) and were followed up over a

period of 2 or 4 weeks. After intraperitoneal anesthesia using ketamine/xylazinehydrochloride (90 μg/7.5 μg (g BW^{-1})), the external carotid artery was cannulated, a blood sample was withdrawn and the alkylglycerol was injected as described above. The concentrations of the alkylglycerols were 100 mM for 1-*O*-pentylglycerol and 75 mM for 2-*O*-hexyldiglycerol. The individual dose of the alkylglycerol was adapted to the respective acute experiments and amounted to 60 mg kg^{-1} BW. Physiological saline and water were added to the alkylglycerols to adjust molality of the final solutions to 320 mosm kg^{-1}. No MTX was given in these experiments in order to evaluate the toxic effects of the alkylglycerols alone. Control animals received intracarotid physiological saline. After drug administration, the external carotid artery was ligated, the catheter removed and the animals awoke rapidly from anesthesia. During the observation periods of 2 and 4 weeks, BW and clinical status were registered daily. At the end of the experiments, the rats were anesthetized again, blood samples were taken, the left ventricle was cannulated and the brain was perfused using 100 ml Ringer's solution followed by 100 ml freshly prepared 4% paraformaldehyde (in PBS buffer). The brain was carefully removed and stored in the fixative for 48 h before further processing.

Toxicological evaluation

Hematological and clinical chemistry parameters were measured twice before alkylglycerol administration (day 0) and at the end of the follow-up period (day 14 or 28). Blood cells were counted by a hematocytometer (Minos STE, ABX, Göppingen, Germany). The hemoglobin concentration was determined photometrically. Serum parameters (Na, K, Ca, protein, aminotransferases (GOT, GPT), lactate dehydrogenase (LDH) and creatinine) were analyzed with a Beckman autoanalyzer (Synchron CX5D, Beckman, München, Germany). Values were compared to those obtained in the control group and to normal values derived from 40 untreated healthy male Wistar rats weighing 290 $\pm$ 40 g used in other experiments.

For histology, whole brains were fixed for 48 h in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned at 1–2 μM. Sections were stained with hematoxylin and eosin or with cresyl violet. Immunohistochemistry was carried out as described previously (Heber *et al*., 2000). Rehydrated, 2-μM-thick sections were incubated with the following monoclonal mouse antibodies: glial fibrillary acid protein (GFAP; Dako, Hamburg, Germany, 1 : 50) and a microglial marker (CD-68, Dako, 1 : 50) diluted in PBS for 2 h at room temperature. For secondary antibodies, we used rabbit anti-mouse IgG (Dako) diluted 1 : 50 in PBS for 45 min at room temperature. Bound secondary antibody was detected by using the alkaline phosphatase–antialkaline phosphatase complex (APAAP; mouse monoclonal, Dako, Hamburg, Germany) diluted 1 : 40 in PBS and incubated for 45 min at room temperature. The alkaline phosphatase activity was visualized by using Astranenfuchsin (Aldrich, Milwaukee, WI, U.S.A.). Apoptotic cell death was assessed by detection of DNA fragmentation using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL)-method as described by Heber *et al*. (2000). All sections were examined by standard light microscopic techniques. An independent blinded investigator performed the analyses.

Statistical evaluation

Data are given as mean $\pm$ s.d. unless otherwise indicated. Mann–Whitney rank sum test was used for statistical analysis. To evaluate the relation between the alkylglycerol concentrations injected and the MTX concentration measured in the brain tissue, Pearson's correlation coefficient was calculated.

Results

In vivo *experiments*

After intracarotid administration of MTX without alkylglycerols, low tissue concentrations were found in the brain parenchyma amounting to 0.39 $\pm$ 0.22 pmol mg^{-1} in the right hemisphere, 0.28 $\pm$ 0.14 pmol mg^{-1} in the left hemisphere and 0.39 $\pm$ 0.25 pmol mg^{-1} in the cerebellum. These concentrations were only slightly above those obtained after intravenous administration of MTX (0.24 $\pm$ 0.13, 0.25 $\pm$ 0.15 and 0.36 $\pm$ 0.14 pmol mg^{-1}, respectively). In contrast to this, the simultaneous intracarotid coinjection of MTX with the different alkylglycerols of interest resulted in a marked increase in the MTX concentration in the brain (Table 1). The accumulation of MTX was found predominantly in the right hemisphere ipsilateral to the bolus injection. The amount of MTX delivered to the brain depended on the chemical structure of the coinjected alkylglycerol. From the results obtained, substantial structure–activity relations could be delineated. The 2-*O*-alkylglycerols were significantly less effective than the respective 1-*O*-derivatives (Table 1). The elongation of the alkyl chain to six carbon atoms resulted in higher effectiveness at the BBB (Table 1). 1-*O*-pentyldiglycerol was only effective exceeding a threshold concentration of 100 mM, whereas all other derivatives were characterized by a concentration-dependent stepwise increase in MTX concentrations in the brain. To avoid flow-induced changes in the MTX transfer to the brain, these experiments were performed using identical flow conditions injecting 800 μl of the alkylglycerol–MTX solution per animal. Thus, higher doses were administered with a higher concentration of the respective alkylglycerol. To evaluate whether the effect of the alkylglycerols was dose-dependent, additional experiments were performed using 1-*O*-pentylglycerol varying either the concentration or the administered dose. After coadministration with a dose of 118 mg kg^{-1} 1-*O*-pentylglycerol, MTX concentrations increased with higher 1-*O*-pentylglycerol concentrations. There was a significant relation between the concentration of 1-*O*-pentylglycerol in the injected solution and the MTX concentration detected in the brain (right hemisphere: $r = 0.98$, $P < 0.001$; left hemisphere: $r = 0.90$, $P < 0.02$; cerebellum: $r = 0.96$, $P < 0.005$; Figure 2a). On the other hand, the increase in the dose of 1-*O*-pentylglycerol (100 mM) was not followed by higher MTX concentrations within the brain (Figure 2b).

The administration of mixtures consisting of MTX and two alkylglycerols at concentrations exerting individually only weak effects on the MTX transfer resulted in a considerable high MTX delivery to the right hemisphere. The coadministration of 1-*O*-pentylglycerol (100 mM) or 1-*O*-hexyldiglycerol (50 mM) with 100 mM 1-*O*-butylglycerol, a derivative exhibiting no effects on the BBB at this concentration, was followed by 3.7- and 4.5-fold higher MTX concentrations as compared to

Table 1 Accumulation of MTX in the rat brain after intra-arterial injection in the absence or presence of different alkylglycerol derivatives at various concentrations

	Injected drugs		*Relative accumulation of MTX in the brain*		
	Conc. of alkylglycerol (mM)	*Dose of alkylglycerol* (mg kg^{-1})	*Right hemisphere*	*Left hemisphere*	*Cerebellum and brain stem*
MTX without alkylglycerol			1±0.6	1±0.5	1±0.6
MTX + 1-*O*-pentylmonoglycerol	300	147	314±222	29.8±17.3	30±11
	200	89	110±112	6.3±3.5	5.5±3.1
	100	46	4.8±4.7	1.8±0.2	1.1±0.4
	75	35	4.2±5.1	1.2±0.4	1.2±0.9
	50	22	2.1±0.6	1.1±0.4	0.9±0.2
MTX + 1-*O*-hexylmonoglycerol	100	50	73±29	5.7±5.6	10±9.2
	75	38	34±27	5.9±3.2	7.3±5.9
	50	25	4.7±2.0	1.3±0.3	1.2±0.3
	20	9	3.7±3.3	0.5±0.1	0.5±0.1
	10*	5	1.6±0.5	1.4±0.1	1.0±0.1
MTX + 1-*O*-pentyldiglycerol	200*	126	120±47	3.0±0.8	6.2±2.4
	100	65	1.1±0.3	0.9±0.3	0.8±0.2
	75	51	1.3±0.8	0.7±0.2	0.7±0.1
	50	36	1.0±0.6	1.5±1.1	1.0±0.5
MTX + 1-*O*-hexyldiglycerol	100	69	1442±226	75±63	226±36
	75	56	205±95	13±10	29±22
	50	36	4.3±1.2	1.7±0.1	1.5±0.5
	25	19	1.5±1.2	0.9±0.0	0.7±0.2
MTX + 2-*O*-hexyldiglycerol	100	79	7.7±4.7	1.3±0.5	1.4±0.0
	75	59	1.2±0.5	1.0±0.4	1.2±0.8
	50	35	0.7±0.1	1.0±0.4	0.9±0.1

MTX (5 mg kg^{-1} BW) was injected into the internal carotid artery. For intracarotid coinjections, different alkylglycerols were used. The concentrations of the alkylglycerols varied from 10 to 300 mM. MTX concentrations after intracarotid administration without alkylglycerol were defined as reference values (=1; control). MTX levels obtained in the different experimental groups were related to the reference concentrations in the corresponding brain area of controls. Values given as means±s.d. (four to seven experiments), *$n=3$ experiments.

the injection without 1-*O*-butylglycerol (Figure 3a). Furthermore, after the injection of 1-*O*-pentylglycerol (50 and 100 mM, respectively) in conjunction with 1-*O*- or 2-*O*-hexyldiglycerol, a 6.2- and a 6.9-fold increase in MTX concentrations was obtained in the right hemisphere (Figure 3b). Thus, apart from increasing the concentrations of the alkylglycerols higher BBB permeability could also be obtained by the simultaneous administration of two different derivatives.

The increase in barrier permeability was rapidly reversible. At 3–120 min after the injection of the alkylglycerol, the initial impermeability of the BBB to MTX was almost restored. Interestingly, the time course of the maxima and the minima of MTX penetration into the CNS after such consecutive injections also depended on the chemical structure of the alkylglycerols (Figure 4). After 2-*O*-hexyldiglycerol and 1-*O*-hexylglycerol, a temporary increase in BBB permeability was observed with a maximum 15 min after the alkylglycerol administration. The permeability returned to baseline values 60 min after 2-*O*-hexyldiglycerol and 120 min after 1-*O*-hexylglycerol. Other derivatives such as 1-*O*-pentylglycerol were most effective with simultaneous MTX administration (Figure 4). The differences in the time course and the duration of the permeabilizing effect of the alkylglycerols allow to control the time to reclosure of the BBB and offer a further instrument to modify the amount of drug delivered to the brain.

The MTX transfer to the brain was also investigated after BBB disruption using 25% mannitol and after bradykinin infusion. The administration of hypertonic mannitol resulted in very high MTX concentrations in the ipsilateral right brain, but also in increased MTX concentrations in the contralateral hemisphere and in the cerebellum as compared to controls receiving only intracarotid MTX infusions ($P<0.01$, Table 2). The concentrations were also significantly higher than those obtained after administration of 120 mM 1-*O*-pentylglycerol or bradykinin infusion ($P<0.05$, Table 2). However, the MTX levels after 25% mannitol showed a considerable interindividual variation within a wide range. An aliquot of 120 mM 1-*O*-pentylglycerol and 75 mM hexyldiglycerol resulted in a more homogeneous accumulation of MTX, which was found only in the ipsilateral hemisphere ($P<0.05$ compared to controls, Table 2). In contrast to this, bradykinin exerted only weak effects at the normal BBB resulting in concentrations not significantly higher than in controls ($P>0.05$, Table 2).

In an additional series of *in vivo* experiments, any untoward effects of the intra-arterial alkylglycerols should be detectable. In the experimental groups, the animals received an intracarotid bolus of either 1-*O*-pentylglycerol (100 mM) or 2-*O*-hexyldiglycerol (75 mM) without concomitant MTX, whereas physiological saline was injected to control animals. Following drug administration, the animals awoke rapidly from anesthesia and no clinical signs of neurological damages were observed. The excellent physical state persisted during the follow-up periods of 14 and 28 days. There were no differences in weight gain between alkylglycerol-treated and control animals and a daily thorough clinical examination revealed

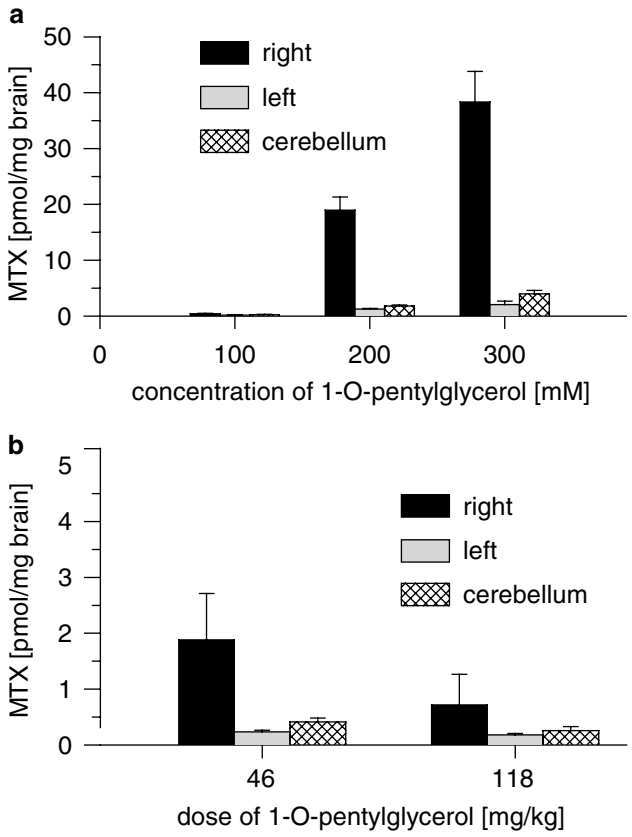

Figure 2 Concentration-dependent increase in MTX delivery to the different brain regions after administration of 1-*O*-pentylglycerol into the right internal carotid artery. (a) Simultaneous intracarotid injection of MTX (5 mg kg^{-1}) and 1-*O*-pentylglycerol (118 mg kg^{-1}). Higher MTX concentrations were found in the different brain regions with increasing the concentration of 1-*O*-pentylglycerol in the injected solution. (b) Simultaneous intracarotid injection of MTX (5 mg kg^{-1}) and 1-*O*-pentylglycerol (100 mM). Increasing the dose of 1-*O*-pentylglycerol does not result in higher MTX transfer to the brain. Right = right hemisphere; left = left hemisphere; $n = 3-5$ animals in each experiment.

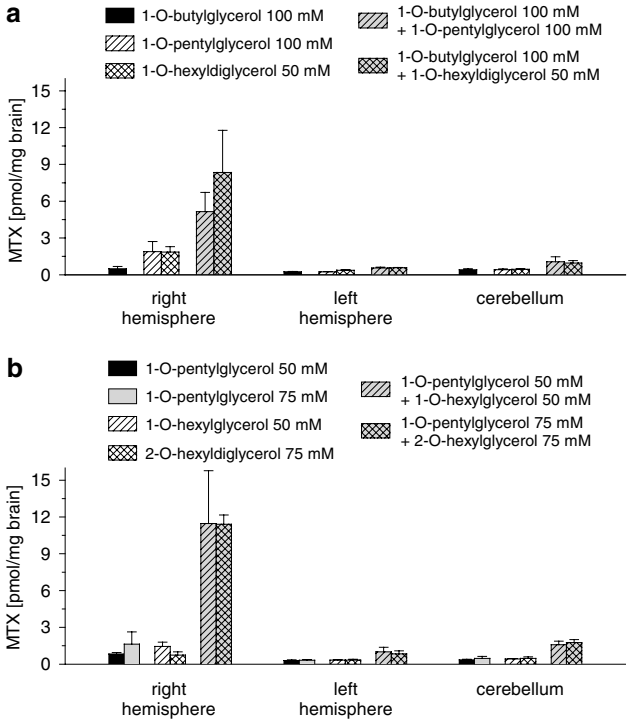

Figure 3 Effect of combinations of alkylglycerols on MTX delivery to the brain. (a) Combination of 1-*O*-pentylglycerol (100 mM) and 1-*O*-hexyldiglycerol (50 mM) with 1-*O*-butylglycerol (100 mM). (b) Combination of 1-*O*-hexylglycerol (50 mM) and 2-*O*-hexyldiglycerol (75 mM) with 1-*O*-pentylglycerol (50 and 75 mM). Alkylglycerols were given simultaneously with MTX (5 mg kg^{-1}) into the right internal carotid artery; $n = 3-5$ animals in each experiment.

no signs of toxicity. The detailed histological analysis of the brains 2 and 4 weeks after the administration of the alkylglycerols demonstrated no pathological changes compared to sham-injected rats. Neither gliosis nor neurodegenerative changes were found even in areas known to be highly susceptible to harmful substances such as the hippocampal formation (Figure 5). There were also no signs of activation of astrocytes or microglia cells as estimated by immunohistochemistry using GFAP and CD-68. Furthermore, alkylglycerol treatment was not found to increase neuronal cell death, as evidenced by pyknotic nuclei in Nissl-stained sections (Figure 5) or by TUNEL staining (data not shown). Hematotoxicity could also be excluded since white blood count, platelet count and hemoglobin concentration showed no changes during the experiments. Furthermore, all clinical chemistry parameters evaluated (serum concentrations of sodium, potassium, calcium, creatinine and protein as well as serum activities of GOT, GPT and LDH) remained unchanged after alkylglycerol treatment and were equal to normal values obtained from 40 untreated healthy rats (data not shown). From these results there was no evidence of severe toxicity on the liver, kidneys or bone marrow.

In vitro *experiments*

Several brain tumor cell lines and human monocytes were incubated with 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol in concentrations up to 200 μM, and the cytotoxic activity of the alkylglycerols was assessed using the WST-1 test. After 24, 48 and 72 h of incubation, no decrease in the cell viability was detected even in the upper concentration range. Moreover, human monocytes were treated for up to 7 days without signs of alkylglycerol-induced cytotoxicity. During alkylglycerol incubation, no morphological changes were observed by a daily morphological evaluation using polarization light microscopy of native cells and light microscopy of methanol-fixed cells stained with Wright's stain (data not shown).

Discussion

Progress in brain tumor chemotherapy is considerably impeded by the BBB and improving delivery of anticancer agents to the brain will play a major role in the therapeutic outcome of brain neoplasms (Kroll & Neuwelt, 1998; Jolliet-Riant & Tillement, 1999). In the past, various attempts have been made to overcome the limiting access of cytotoxic drugs to the brain, and a transient opening of the BBB in humans has been achieved by intracarotid infusion of hypertonic mannitol solutions (Dahlborg *et al*., 1998) or of bradykinin analogs (Cloughesy *et al*., 1999). However, there is no widespread use of either of these methods in clinical neuro-oncology so far.

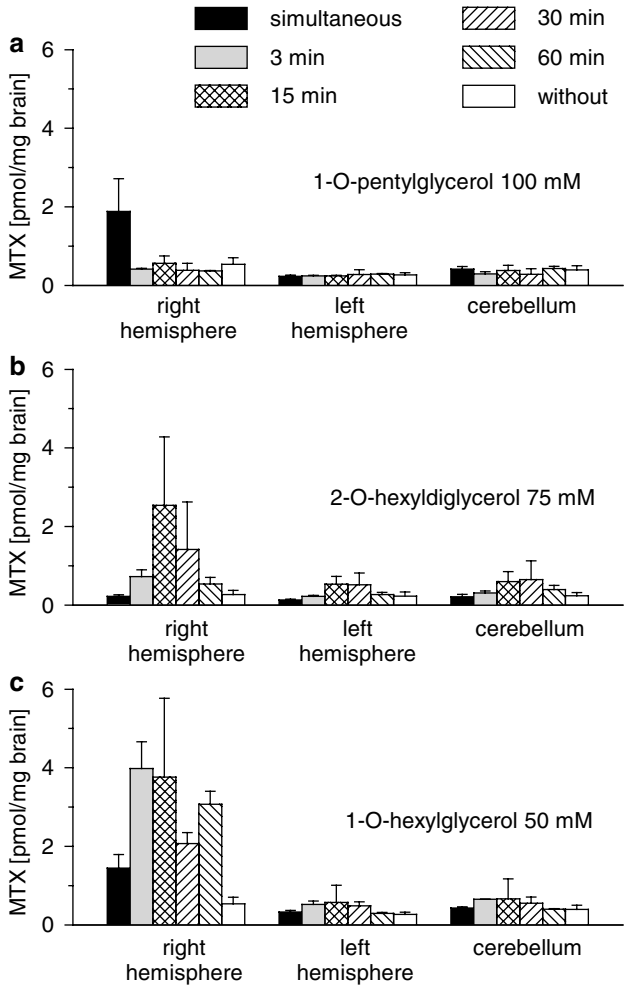

Figure 4 Duration of the alkylglycerol-induced barrier opening. Concentrations of MTX in the brain after intracarotid injection (5 mg kg^{-1}) via the right internal carotid artery simultaneously or separately with (a) 1-*O*-pentylglycerol (100 mM), (b) 2-*O*-hexyldiglycerol (75 mM), and (c) 1-*O*-hexylglycerol (50 mM).

Recently, we described for the first time that the intracarotid administration of alkylglycerols is followed by a strong and reversible increase in BBB permeability to various drugs (Erdlenbruch *et al*., 2000a; 2002). In the present work, the high effectiveness and the low toxicity of short-chain alkylglycerol derivatives are shown and several instruments to modulate the extent of BBB permeability are identified.

The opening of the barrier mediated by these short-chain derivatives was a short-lasting incident with baseline values being restored within 120 min at the latest. The window of barrier opening depended not only on the chemical structure of the alkylglycerol but also on the concentration administered. 1-*O*-pentylglycerol (100 mM) was only effective if administered simultaneously with MTX (see Figure 3). The consecutive injections of 1-*O*-pentylglycerol and MTX have been shown to be ineffective. In earlier experiments with higher concentrations of 1-*O*-pentylglycerol (300 mM), increased concentrations of MTX were found in the brain up to an interval of at least 15 min between the injections of 1-*O*-pentylglycerol and MTX (Erdlenbruch *et al*., 2000a). However, despite the rapid reversibility of the 1-*O*-pentylglycerol-mediated effect, the present data demonstrate that the simultaneous administration of alkylglycerol and drug will not necessarily be followed by the highest drug transfer to the brain. After a two-timed injection of 1-*O*-hexylglycerol and 2-*O*-hexyldiglycerol, a transient increase in MTX delivery was found in the ipsilateral hemisphere. Thus, there are several instruments suited for the control of drug transfer across the BBB as the skilful selection of the alkylglycerol derivative, its concentration, and the well-timed injection of the chemotherapeutic agent. Moreover, the extent of MTX delivered to the brain could also be modified by the administration of a mixture of two different alkylglycerols. The combinations of alkylglycerols represent a further strategy for the regulation of the increased permeability at the BBB.

Opening of the barrier because of osmotic shrinkage of the cerebral endothelium does not occur until a threshold concentration of mannitol in the infusate has been exceeded (Rapoport *et al*., 1980; Hiesinger *et al*., 1986; Blasberg & Groothuis, 1991), and the use of effective concentrations as 1.4 M mannitol resulted in a very marked increase in the delivery of MTX (Kroll *et al*., 1998; see Table 2). Whereas the hyperosmolar disruption of the BBB is characterized by a relative lack of modulating factors that allows regulation of the access of drugs to the brain, the alkylglycerol derivatives investigated in the present study allow the varying of the delivery of MTX within a wide range. The administration of high concentrations of the 1-*O*-derivatives was as effective as

Table 2 Concentration of MTX in the rat brain after intracarotid infusion in the absence or presence of hypertonic mannitol, 1-*O*-pentylglycerol, 2-*O*-hexyldiglycerol or bradykinin

Drugs administered	*Concentration of MTX* (pmol mg^{-1} brain)		
	Right hemisphere	*Left hemisphere*	*Cerebellum and brain stem*
Controls MTX (5 mg kg^{-1})	0.45±0.04	0.21±0.04	0.28±0.04
Mannitol 1.4 M + MTX (5 mg kg^{-1})	50.8*,**±19.7	4.65*,**±2.26	5.55*,**±2.24
1-*O*-Pentylglycerol 120 mM + MTX (5 mg kg^{-1})	2.35***±0.98	0.27±0.05	0.39±0.08
2-*O*-hexyldiglycerol 75 mM + MTX (5 mg kg^{-1})	0.80***±0.14	0.26±0.02	0.40±0.02
Bradykinin 10 μg kg^{-1} min^{-1} + MTX (5 mg kg^{-1})	0.72****±0.08	0.36±0.03	0.46±0.03

MTX (5 mg kg^{-1} BW) was continuously infused into the right internal carotid artery over 15 min. Control animals received no blood – brain barrier modification. Mannitol 1.4 M, 1-*O*-pentylglycerol 120 mM and 2-*O*-hexyldiglycerol 75 mM were given immediately before starting the MTX infusion. Bradykinin was administered at a rate of 10 μg kg^{-1} min^{-1} for 15 min. The MTX-infusion was started 5 min after the start of bradykinin. Values given are means±s.e.m., $n=6–8$ experiments in each group. *$P<0.01$, mannitol *versus* control and mannitol *versus* bradykinin; **$P<0.05$ mannitol *versus* 1-*O*-pentylglycerol, ***$P<0.05$ 1-*O*-pentylglycerol and 1-*O*-hexyldiglycerol *versus* control, ****$P>0.05$ bradykinin *versus* control.

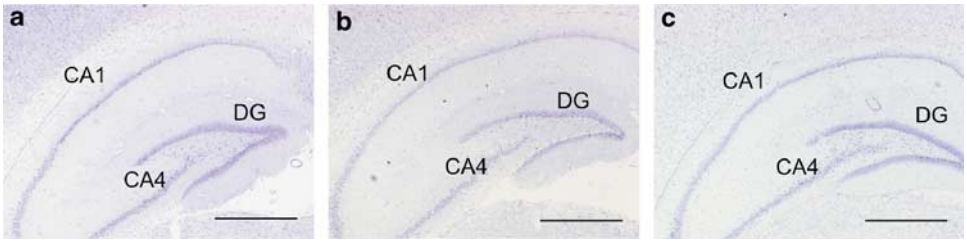

Figure 5 Lack of histological alterations after intra-arterial alkylglycerols. An intracarotid bolus injection of 1-*O*-pentylglycerol (100 mM, $n=6$), 2-*O*-hexyldiglycerol (75 mM, $n=6$) or physiological saline ($n=6$) was given to normal rats. At 14 or 28 days after the treatment, brains were removed and histological evaluation was performed. Sections depicted show the dentate gyrus (DG) and pyramidal cells of the CA1 and CA4 region of the hippocampal formation stained by Nissl's stain. (a) Control (NaCl 0.9%), (b) 1-*O*-pentylglycerol (100 mM), (c) 2-*O*-hexyldiglycerol (75 mM); scale bars (a–c) = 1 mm.

25% mannitol. As expected from data published by Inamura *et al*. (1994), the intracarotid infusion of bradykinin induced only marginal effects at the cerebral vasculature of the normal brain.

There has been a controversy in the literature whether osmotic BBB disruption or bradykinin- and RMP-7-mediated barrier opening is more suited to treat brain tumors (Kroll *et al*., 1998). Depending on the tumor model used, intracarotid administration of bradykinin and RMP-7 showed a weak but highly selective increase in blood–tumor permeability (Inamura *et al*., 1994; Nomura *et al*., 1994; Matsukado *et al*., 1996). In contrast to this, hyperosmolar BBB disruption has been described to exert strong effects in tumor, but higher effectiveness was seen in brain adjacent to tumor and tumor-free surrounding brain. As mannitol is more effective in increasing delivery to the normal brain distant to tumor than to the tumor itself, it raises the potential for toxicity (Inoue *et al*., 1987; Barnett *et al*., 1995; Kroll *et al*., 1998). Consequently, the important question arises whether the entire vascular bed of the infused hemisphere needs to be disrupted or a highly selective disruption of the tumor's vasculature is more appropriate for effective chemotherapeutic treatment. Intracarotid alkylglycerols are able to improve drug delivery to both the brain tumor itself and to the surrounding ipsilateral normal brain including infiltrative tumor cells. No preference of the alkylglycerols has been found in favor of the normal tumor-free brain after the administration of MTX to C6 glioma-bearing rats in the presence of 1-*O*-pentylglycerol (Erdlenbruch *et al*., 2000a). In contrast, a slight tumor-to-brain selectivity of the permeabilizing effect has been described in the same tumor model after treatment with erucylphosphocholine in the presence of 1-*O*-pentylglycerol (Erdlenbruch *et al*., 2002). In preliminary experiments, using intracarotid chemotherapy of glioma-bearing rats with cisplatin in conjunction with 1-*O*-pentylglycerol, a significant increase in survival rate was observed (unpublished data).

Concerning the extent of untoward effects, the rapid reversibility of the alkylglycerol-mediated chemical opening of the BBB might be of advantage in a clinical setting. Due to the short latency to reclosure of the opened barrier there is only a short period during which potentially neurotoxic agents may enter the brain and may cause toxicity. In view of the recently published data from Siegal *et al*. (2000), who have shown that the barrier opening after hypertonic mannitol lasts for approximately 6–8 h, this would be a putative superiority of this new experimental approach over the osmotic BBB disruption. Furthermore, the administration of hypertonic mannitol was associated by a transient but strong decrease in arterial blood pressure, while the alkylglycerols induced no changes in circulation parameters (data not shown).

No signs of acute toxicity have been described using a multitude of intracarotid alkylglycerols in a lower concentration range (Erdlenbruch *et al*., 2000a). However, *in vitro* investigations demonstrated hemolytic effects during incubation of erythrocytes with derivatives composed of alkyl chains longer than six carbon atoms (heptyl and octyl groups, unpublished data). Since those potentially toxic compounds have also shown disproportionate strong effects at the BBB *in vivo*, the further evaluation of long-term toxicity of alkylglycerols in the present study was restricted to substances with properties favorable for a putative clinical application. Up to 4 weeks after intracarotid 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol, there were neither clinical nor laboratory signs of alkylglycerol-induced toxicity. Moreover, the neuropathological evaluation 2 and 4 weeks after the injection revealed no histological abnormalities within the CNS compared to control animals. Biodistribution studies using radiolabeled 1-*O*-pentylglycerol (^{3}H and ^{14}C) have shown that no accumulation of 1-*O*-pentylglycerol took place in the brain parenchyma. Pharmacokinetic data suggested a rapid renal elimination of 1-*O*-pentylglycerol within a few hours after both intravenous and intra-arterial bolus injections. Analyses performed 270 min after administration of 1-*O*-pentylglycerol pointed out that radioactivity in the hemisphere ipsilateral to the bolus injection was as low as in the contralateral hemisphere and levels were equal to those found in plasma and most other organs (unpublished data). Hematological and clinical chemistry parameters in the same experiments showed no change up to 3 days after 1-*O*-pentylglycerol. These data are consistent with the lack of toxic side effects in the present study.

Taken together, short-chain alkylglycerols have been shown to increase the transfer of MTX to the brain and several instruments have been described to control the alkylglycerol-mediated increase in barrier permeability. *In vitro* and *in vivo* assessment revealed that this new concept of BBB opening is associated with no toxic effects at therapeutic levels. Thus, intracarotid alkylglycerols represent a new therapeutic procedure to overcome the limited access of therapeutic agents to the CNS. Further experiments using different glioma models

have to confirm preliminary results demonstrating a benefit of intra-arterial chemotherapy in conjunction with alkylglycerols in glioma-bearing rats and to clarify whether durable responses can be achieved.

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