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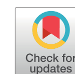
Research paper

Targeted brain delivery of methotrexate by glutathione PEGylated liposomes: How can the formulation make a difference?



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ABSTRACT

The purpose of this study was to quantitatively investigate how conjugation of GSH to different liposomal formulations influence the brain delivery of methotrexate (MTX) in rats. GSH-PEG liposomal MTX based on hydrogenated soy phosphatidylcholine (HSPC) or egg yolk phosphatidylcholine (EYPC) and their corresponding PEG control liposomes were prepared. The brain delivery of MTX after intravenously administering free MTX, four liposomal formulations or free MTX + empty GSH-PEG-HSPC liposomes was evaluated by performing microdialysis in brain interstitial fluid and blood. Compared to free MTX with a steady-state unbound brain-to-plasma concentration ratio ($K_{p,uu}$) of 0.10, PEG-HSPC liposomes did not affect the brain uptake of MTX, while PEG-EYPC liposomes improved the uptake ($K_{p,uu}$ 1.5, $p < 0.05$). Compared to PEG control formulations, GSH-PEG-HSPC liposomes increased brain delivery of MTX by 4-fold ($K_{p,uu}$ 0.82, $p < 0.05$), while GSH-coating on PEG-EYPC liposomes did not result in a further enhancement in uptake. The co-administration of empty GSH-PEG-HSPC liposomes with free MTX did not influence the uptake of MTX into the brain. This work showed that the brain-targeting effect of GSH-PEG liposomal MTX is highly dependent on the liposomal formulation that is combined with GSH, providing insights on formulation optimization of this promising brain delivery platform.

1. Introduction

A critical factor limiting the treatment of brain diseases is the incapacity of drugs to reach the brain in sufficient quantities to be effective, due to the highly restrictive blood-brain barrier (BBB) [1,2]. One possibility to enhance blood-to-brain drug delivery may be to formulate drugs in nanocarriers like liposomes. An important reason why liposomal brain delivery is expected to be promising is that it is possible to functionalize the surface of the liposomes with BBB-targeting ligands to potentially achieve brain-targeted delivery [3,4]. By choosing a ligand (e.g., antibodies, peptides) specific for proteins or receptors expressed at the BBB, the liposomes may take advantage of endogenous BBB-crossing mechanisms and ultimately facilitate drug delivery into the brain parenchyma [5].

Nutrient transporters like the glutathione transporter, which is expressed preferentially in the central nervous system (CNS) and the BBB, is present in all mammalian species and may be utilized to improve brain delivery [6–8]. Glutathione (GSH) is an endogenous tripeptide with antioxidant-like properties [9,10]. Additionally, GSH possesses a well-established and good safety profile when being administered exogenously [10]. 2-BBB has developed the G-Technology® which utilizes GSH as a targeting ligand conjugated onto PEGylated (PEG) liposomes. GSH-PEG liposomes have been shown to safely improve brain delivery of various therapeutic agents including small molecular drugs [9,11], peptide [12] and single domain antibody fragments [13] in animal models.

GSH-PEG liposomes have been shown to be more beneficial in delivering drugs into the brain than non-targeted PEG liposomes. For example, Rip et al. demonstrated that GSH-PEG liposomal ribavirin led to up to 5-fold higher unbound drug concentration in the brain compared to PEG control liposomes, while having similar systemic exposure [14]. Another study showed that the unbound brain level of a fluorescence tracer, carboxyfluorescein was increased 4-fold when being delivered with GSH-PEG liposomes compared to plain PEG liposomes, while the total plasma levels were similar [10]. However, the brain-targeted effect was not observed for GSH-PEG liposomal DAMGO, an opioid peptide, compared to PEG liposomal DAMGO [15]. Apart from different payloads, another critical factor that may contribute to the

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distinct effect of GSH-PEG liposomal formulations on brain delivery between these studies is the different liposomal formulations that are combined with GSH. In cases where GSH-PEG liposomes were superior to PEG controls, the formulations contained hydrogenated soy phosphatidylcholine (HSPC), while in the study where GSH-PEG liposomes showed no advantage over PEG control liposomes, egg-yolk phosphatidylcholine (EYPC) was the main phospholipid used in the formulation. Also, our previous study has shown that different formulations of PEG liposomal methotrexate (MTX) based on HSPC or EYPC, can result in very different brain delivery of MTX [16]. EYPC based formulation increased brain uptake of MTX three-fold, while the HSPC based one had no impact on the uptake at all compared with free MTX. However, there are no systematic and quantitative studies testing whether or not formulations with different phospholipids have an impact on the outcomes of brain-targeted delivery of GSH-PEG liposomes.

In the current study, we therefore used MTX as a model drug, being suitable for microdialysis and also a good substrate for liposome-encapsulation with superior stability both *in vitro* and *in vivo*. It explores whether the brain-targeting effect of GSH-PEG liposomes depends on the liposomal formulation, providing insights into the formulation optimization of this promising brain delivery platform. Two formulations of GSH-PEG liposomal MTX based on HSPC and EYPC as well as their corresponding PEG control liposomes were produced. The purpose of this study was to quantitatively investigate how the conjugation of GSH may influence brain delivery of MTX in combination with different liposomal formulations in rats. To achieve this goal, continuous microdialysis sampling in both blood and striatum combined with regular blood sampling were performed, which enabled the separation of released, active drug from drug remaining liposomally encapsulated, and thereby being able to describe the multiple *in vivo* processes present.

2. Materials and methods

2.1. Materials

MTX solution for injection (100 mg/mL) was obtained from Leiden University Medical Center Pharmacy (Leiden, The Netherlands). Deuterated MTX (MTX-D3) was purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogenated soy phosphatidylcholine (HSPC) and egg-yolk phosphatidylcholine (EYPC) were obtained from Lipoid (Cham, Switzerland). Cholesterol and reduced glutathione (GSH) was purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-conjugated polyethylene glycol MW 2000 (mPEG$_{2000}$-DSPE) and DSPE-PEG$_{2000}$-maleimide were purchased from Laysan Bio Inc. (Alabama, USA). The Ringer solution was prepared in-house, containing 145 mM NaCl, 0.6 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 0.1 mM ascorbic acid in 2.0 mM phosphate buffer (pH 7.4). Acetonitrile (ACN) and formic acid (FA) were of analytical grade (Merck, Darmstadt, Germany). The water was purified with a Milli-Q Academic system (Millipore, Bedford, MA, USA).

2.2. Liposome preparation and characterization

Two formulations of GSH-PEG liposomal MTX containing either HSPC or EYPC as well as their corresponding PEG control liposomes without GSH were prepared using an ethanol injection method with post-insertion of GSH-PEG micelles or PEG micelles. In brief, the lipids including 100 mM phospholipids, either HSPC or EYPC, 66 mM cholesterol and 1.7 mM mPEG$_{2000}$-DSPE (1 mol%), were dissolved in 4.8 mL absolute ethanol and then mixed with 15.2 mL MTX solution (50 mg/mL, diluted from the original MTX injection solution with saline) at 60 °C. The formed liposomes were extruded step-wise through 400/200 nm, 200/200 nm, 200/100 nm and 100/100 nm Whatman filters (Instruchemie, Delfzijl, the Netherlands) to reduce and unify the particle size. GSH and DSPE-PEG$_{2000}$-maleimide (4 mol%) were incubated at a 1.5:1 M ratio for 2 h at room temperature to form GSH-PEG-DSPE micelles. PEG-DSPE micelles were also made by incubating mPEG2000-DSPE (4 mol%) under the same condition. GSH-PEG-DSPE or PEG-DSPE micelles for PEG control liposomes were post-inserted to the liposomes at 60 °C for 2 h. The final total molar percentage of PEG in the liposomes was 5%, with 4% GSH-PEG-DSPE and 1% PEG-DSPE for GSH-PEG liposomes, or with 4% PEG-DSPE post-inserted and 1% PEG-DSPE pre-inserted for PEG control liposomes. After post-insertion of the micelles, the liposomes were purified via ultrafiltration using a Pellicon XL 50 Cassette ultrafiltration column equilibrated with saline on a Cogent® μScale Tangential Flow Filtration System (Merck Millipore) to remove non-encapsulated MTX and excess GSH. The purified liposomes were sterile filtered using 0.2 μm filters, and aliquots were stored at 4 °C until further use.

The size of the liposomes was measured using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). The encapsulated MTX was determined using an HPLC-UV assay after releasing the drug from the liposomes with ACN. In short, chromatographic separation was carried out on a Xbridge C18 column (150 × 4.6 mm, 3.5 μm) (Waters, CA, USA) using a Shimadzu 20A ultra-fast liquid chromatography system (Shimadzu, Kyoto, Japan) under isocratic elution with a flow rate of 1 mL/min and the column temperature of 23 °C. The mobile phase consisted of ACN: buffer (10:90, v/v) of which the buffer is the mixture of disodium hydrogen phosphate and citric acid (63:37, v/v). The UV wavelength for detection was set at 302 nm. The lipid levels in the liposomes were quantified using HPLC combined with evaporative light-scattering detection (ELSD) (Alltech, the Netherlands). A Kinetex C18 (150 × 4.6 mm, 2.6 μm, Phenomenex) column equipped with a guard column was used for analysis of HSPC, EYPC, cholesterol, GSH-PEG-DSPE and PEG-DSPE. The column temperature was set to 45 °C. All lipids were chromatographically separated within 30 min under a gradient elution of mobile phase A (0.1 M ammonium acetate, pH 6.0) and 90–100% mobile phase B (methanol) at a flow rate of 1.5 mL/min. For the ELSD, the nitrogen gas flow was set to 1.5 mL/min and temperature in the drift tube was 80 °C.

2.3. In vitro stability of liposomes

The liposome stability in both phosphate buffer saline (PBS) and rat plasma was evaluated. For the experiment, 10 μL solution of each liposomal formulation was diluted with 40 μL of either PBS or rat plasma and then incubated at 37 °C for 48 h. During the incubation, samples were taken at 0, 1, 2, 4, 8, 24 and 48 h. The collected sample was diluted with 150 μL PBS before loading 100 μL of the diluted sample onto a Zebaspin desalting column (Thermo Scientific, Rockford, IL, USA) equilibrated with PBS. After removal of potentially released MTX, ACN was added to the purified sample to release the encapsulated MTX. The concentrations of MTX were analyzed using the abovementioned HPLC-UV method. The amount of liposomal MTX at each time point was expressed as the percentage of the value at time 0 (T0).

2.4. Animals

Male Sprague Dawley rats, obtained from Taconic (Lille Skensved, Denmark) were used throughout the experiment. Before the experiments, the rats were housed in groups and acclimatized for 7 days under temperature- and humidity-controlled conditions in a 12 h light/dark cycle with unlimited access to food and water. The rats weighed 230–290 g on the day of the experiment. The experimental protocols and animal procedures were approved by the Uppsala Regional Animal Ethics Committee, Uppsala, Sweden (C13/14).

2.5. Surgery

The rats were anesthetized during the surgery by inhaling 2.5% isoflurane (Isoflurane Baxter®, Baxter Medical AB, Kista, Sweden), combined with 1.5 L/min oxygen and 1.5 L/min nitrous oxide. The

body temperature was kept at 38 °C using a heating pad (CMA/150 temperature controller, CMA, Stockholm, Sweden). Pre-heparinized (100 IU/mL heparin in saline) PE-50 cannulas (MicLev, Malmö, Sweden) were inserted into the left femoral artery for blood sampling and into the left and right femoral vein for drug/liposomes administration. In order to measure the released, unbound MTX concentrations in blood, a flexible CMA/20 microdialysis probe with 10 mm polyarylethersulfone (PAES) membrane and 20 kD cut-off (CMA, Stockholm, Sweden) was implanted into the right jugular vein and fixed to the pectoral muscle by two sutures. For sampling in brain interstitial fluid (ISF), a CMA/12 guide cannula was initially inserted into the striatum (position: 2.7 mm lateral and 0.8 mm anterior to the bregma and 3.8 mm ventral to the brain surface) using a stereotaxic instrument (David Kopf Instruments, Tujunga, USA) and secured to the skull with a screw and dental cement (Dentalon® Plus Heraeus, Germany). Subsequently, the guide cannula was carefully replaced by a CMA/12 probe with 3 mm PAES membrane and 20 kD cut-off (CMA, Stockholm, Sweden). All catheters were passed subcutaneously to the posterior surface of the neck. After surgery, the rat was individually placed in a CMA/120 system for freely moving animals and allowed to recover for 24 h before the start of the experiment, which was performed on awake animals.

2.6. *In vivo quantitative study design*

On the experiment day, the microdialysis probes were perfused with Ringer solution containing MTX-D3 as the recovery calibrator. The concentrations of MTX-D3 in brain and blood perfusate were 20 and 200 ng/mL, respectively. The rate of perfusion was maintained at 0.5 μL/min using a CMA/100 precision infusion pump (CMA, Stockholm, Sweden). In order to stabilize the system, the perfusion started at least 90 min before the beginning of drug administration. Fluorinated ethylene propylene (FEP) tubings (CMA, Stockholm, Sweden) were used as both inlet and outlet tubings. The *in vivo* probe recovery was determined throughout the whole experiment based on retrodialysis [17].

To compare the pharmacokinetic (PK) profiles between free MTX and liposomal formulations, the rats were randomly assigned to five groups: GSH-PEG-HSPC liposomes (referred to as "GSH-PEG-HSPC") (n = 7), PEG-HSPC liposomes (referred to as "PEG-HSPC") (n = 7), GSH-PEG-EYPC liposomes (referred to as "GSH-PEG-EYPC") (n = 7), PEG-EYPC liposomes (referred to as "PEG-EYPC") (n = 7) and free MTX (n = 4). For the liposomal groups, the rats received 30-min intravenous administration of the four formulations at the same MTX dose (15 mg/kg) through the left femoral vein using a Harvard 22 pump (Harvard Apparatus Inc., Holliston, MA). In order to achieve comparable steady-state unbound MTX concentrations in plasma after administering free MTX and liposomal formulations, a pilot study was performed to determine the doses needed in the "free drug group". The free MTX was intravenously administered as a loading dose of MTX (7.2 μg/min/kg) during the initial 0.5 h, followed by a 9.5 h constant infusion (6 μg/min/kg). After observing an increased unbound brain-to-plasma concentration ratio after administration of GSH-PEG-HSPC compared to PEG-HSPC and free MTX in the pilot study, a co-administration group was additionally included (n = 4). In this group, free MTX (same dose regimen as the "free drug group") and empty GSH-PEG-HSPC liposomes (same HSPC dose as GSH-PEG-HSPC) were simultaneously injected into the left and right venous catheters, respectively. The reason for adding this group was to examine if GSH-PEG-HSPC liposomes themselves may influence the BBB integrity, function and thereby the uptake of MTX.

The microdialysate fractions were collected from blood and brain ISF every 30 min (~15 μL per fraction) in polypropylene vials (AgnThos, Lidingö, Sweden), starting from 1 h before the infusion started until 10 h after the beginning of the infusion. All the sampling vials were weighed before and after sample collection to monitor the flow through the probe during the experiment. The midpoint time in each collection interval was used to plot the concentration-time curves. Blood (~200 μL) was withdrawn into heparinized polypropylene tubes at pre-dose, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10 and 24 h after the start of the infusion. The collected blood samples were immediately centrifuged at 10000 rpm (7200*g*) for 5 min, after which the plasma was transferred to clean polypropylene tubes. All microdialysis and plasma samples were stored at −20 °C until further analysis.

2.7. *Sample analysis*

An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was employed to quantify MTX and MTX-D3 in microdialysate and plasma. The plasma sample obtained from the liposomal groups (2.5 μL) was first diluted 20-fold with blank rat plasma, followed by protein precipitation with 150 μL of ACN containing 1% formic acid (FA) and MTX-D3 as the internal standard (IS). The plasma sample (50 μL) from the free MTX and co-administration groups was directly precipitated by adding 150 μL of IS- and FA-containing ACN. After centrifugation for 3 min at 13,000 rpm, an aliquot of 10 μL of the supernatant was diluted with 400 μL of 0.01% FA in 10% ACN (mobile phase A), before injecting 5 μL onto the UPLC-MS/MS system. For brain and blood microdialysate, a volume of 10 μL sample was mixed with 90 μL mobile phase A. Then, 5 μL of the diluted sample was injected onto the UPLC-MS/MS system.

Chromatographic separation was performed on an ACQUITY BEH C18 column (50 × 2.1 mm, 1.7 μm) protected by an ACQUITY BEH C18 guard column (10 × 2.1 mm, 1.7 μm) (Waters, CA, USA) using a Waters ACQUITY UPLC system (Waters, CA, USA). The mobile phase consisted of 0.01% FA in 10% ACN (A) and 0.01% FA in 90% ACN (B). The gradient elution started at 0% B for 1 min, then increased linearly to 90% B within 1 min and kept at 90% B for 0.5 min, before returning to 0% B for equilibration. The flow rate was 0.3 mL/min and the total run time was 3 min. A Quattro Ultima triple quadrupole mass spectrometer was used for MS/MS detection (Waters, Milford, MA, USA), and MassLynx software version 4.1 (Waters, Milford, MA, USA) was utilized for data acquisition and processing. The detection of MTX and MTX-D3 was performed in a positive electrospray mode using multiple reaction monitoring transitions of m/z 455.2 → 308.0 for MTX and m/z 458.3 → 311.0 for MTX-D3. The linearity range for plasma samples was between 5 and 20000 ng/mL. For microdialysis samples, the standard curves for both MTX and MTX-D3 ranged from 0.1 to 500 ng/mL. By using $1/x^2$ weighing, the coefficient of determination (R^2) for all the standard curves was higher than 0.99. The precision and accuracy for quality controls were below 15% coefficient of variation (CV) in all runs.

2.8. *Data analysis*

The *in vivo* recovery for each microdialysis probe was calculated as

$$\text{Recovery} = \frac{C_{\text{calibrator,in}} - C_{\text{calibrator,out}}}{C_{\text{calibrator,in}}} \quad (1)$$

where $C_{\text{calibrator,in}}$ is the concentration of MTX-D3 in the microdialysis perfusate determined in triplicates before and after the experiment and $C_{\text{calibrator,out}}$ is the average concentration of MTX-D3 in the collected dialysate during the experiment.

Adsorption of both MTX and MTX-D3 to the probes or tubings was negligible. The recovery of the microdialysis probes was stable throughout the experiments. The average recovery was 0.09 ± 0.05 and 0.57 ± 0.14 for the brain and the blood probes, respectively.

The unbound concentration (C_u) of MTX in brain ISF and plasma was then calculated from Eq. (2) as

$$C_u = \frac{C_{\text{dialysate}}}{\text{Recovery}} \quad (2)$$

where $C_{\text{dialysate}}$ is the concentration of MTX in the collected dialysate

and recovery is the average of individual recovery measured from all brain or blood probes.

According to non-compartmental analysis, the clearance (CL) of MTX after administration of free MTX, liposomal MTX or free MTX + empty liposomes was estimated in two ways:

For the free MTX and the co-administration groups:

$$CL = \frac{R_0}{C_{tot,ss,plasma}} \tag{3}$$

For the liposomal groups:

$$CL = \frac{Dose}{AUC_{0\to\infty}} \tag{4}$$

where R_0 is the infusion rate of free MTX with or without empty liposomes and $C_{tot,ss,plasma}$ is the steady-state plasma concentration of MTX. The $AUC_{0\to\infty}$ is the area under the concentration-time curve of total MTX (released and encapsulated) in the four liposomal groups, calculated using the linear trapezoid method. The residual areas for $AUC_{0\to\infty}$ were calculated as C_{last}/λ_z, where C_{last} is the concentration measured at 24 h and λ_z is the terminal rate constant estimated from the slope of the 4 last concentrations measured. For the liposomal groups, the terminal half-life ($t_{1/2}$) and the volume of distribution (V_d) were calculated as ln (2)/λ_z and CL/λ_z, respectively.

The unbound fraction of MTX in plasma (f_u) after administration of free MTX or free MTX + empty liposomes was calculated as $C_{u,ss,plasma}/C_{tot,ss,plasma}$, where $C_{u,ss,plasma}$ and $C_{tot,ss,plasma}$ represent the concentration of unbound and total MTX in plasma at steady state, obtained from the microdialysis and plasma samples collected 1–10 h after the start of the administration. For the liposomal groups, $C_{u,ss,plasma}/C_{tot,ss,plasma}$ was also calculated based on the microdialysis and plasma samples collected 8–10 h after infusion started to describe the extent of *in vivo* drug release.

The brain delivery of MTX was described by the ratio of unbound drug in the brain to that in plasma at steady state, $K_{p,uu}$ [18,19], calculated as:

$$K_{p,uu} = \frac{C_{u,ss,brain}}{C_{u,ss,plasma}} \tag{5}$$

where $C_{u,ss,brain}$ and $C_{u,ss,plasma}$ represent the steady-state concentrations of unbound MTX in brain and plasma, respectively, obtained from the brain and plasma microdialysate collected 8–10 h after the start of the infusion.

Statistical analyses were performed using R version 3.5.0 [20] using the multicomp package [21]. Differences between the groups were assessed using a linear model with the log-transformed ratio as the dependent variable. Assumptions of constant variance and normality of the residuals were checked visually using quantile-quantile plots of the residuals versus quantiles from a normal distribution and plots of residuals versus fitted values. Multiplicity adjusted *p*-values were calculated taking the correlations of the individual test statistics into account which is more efficient than the usual Bonferroni adjustment [22]. A $^*p < 0.05$ was considered to be statistically significant.

3. Results

For all liposomal formulations, the size, polydispersity index (PDI), encapsulated MTX and lipids concentrations were measured and are presented in Table 1. All formulations were comparable in size and MTX contents. For both GSH-PEG-HSPC and GSH-PEG-EYPC liposomes, the presence of GSH-PEG lipid confirmed successful conjugation of GSH on the surface of the liposome. After the incubation in either PBS or rat plasma for 48 h, no significant differences in the levels of liposomally encapsulated MTX were observed for any of the liposomal formulation relative to their initial values (Fig. 1), suggesting that all the formulations had excellent stability *in vitro*.

In the free MTX (Fig. 2A) and co-administration group (Fig. 2D), a steady state of total plasma concentration was reached within 1 h after a loading dose was given. The total plasma CL of MTX was comparable regardless of whether or not empty GSH-PEG-HSPC was co-administered together with the free drug (Table 2).

After 30-min infusion of the four liposomal formulations at the same doses, the total plasma concentrations of MTX were similar independent of the formulation administered and were very stable up to 10 h with long half-lives ranging from 22 to 27 h (Fig. 2B, C, E and F and Table 2). Also, the CL of total MTX in plasma was naturally much lower than that observed when administering free MTX either with or without empty GSH-PEG-HSPC liposomes.

The unbound fraction of MTX in plasma was similar when free MTX was administered by itself or co-administered with empty GSH-PEG-HSPC liposomes (Table 2). The total plasma concentration of MTX, including both released and liposomally encapsulated MTX, was 717–4330 times higher than the unbound MTX concentration in plasma in the liposomal groups (Table 3, Fig. 2B, C, E, and F). The four liposomal formulations released MTX to different extents, as reflected by the different ratios of unbound to total MTX in plasma. In general, the extent of MTX release in plasma was significantly greater from EYPC-based than from HSPC-based formulations ($p < 0.05$). However, there

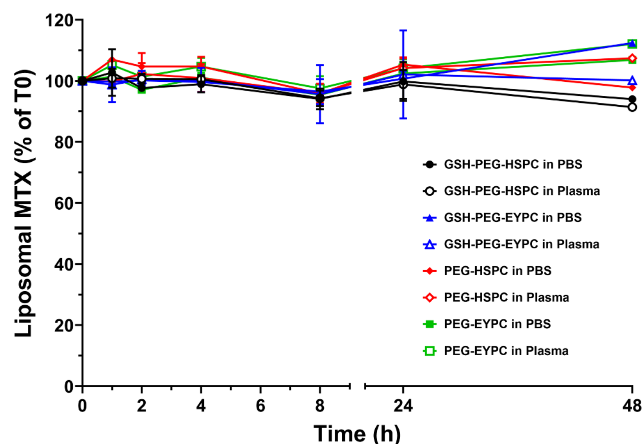

Fig. 1. *In vitro* stability of liposomal MTX formulations in PBS or rat plasma at 37 °C. (n = 3).

Table 1
Characteristics of liposomal MTX.

	Size[a] (PDI)	Concentration (mg/mL)					
		MTX	HSPC	EYPC	Cholesterol	PEG	GSH-PEG
GSH-PEG-HSPC liposomes	126.3 (0.074)	4.8	27.3	–	7.7	1.5	9.4
GSH-PEG-EYPC liposomes	122.6 (0.089)	5.4	–	36.0	7.4	2.2	9.3
PEG-HSPC liposomes	126.3 (0.064)	5.5	37.4	–	7.2	14.2	–
PEG-EYPC liposomes	119.1 (0.105)	5.8	–	54.6	7.0	20.5	–

[a] Size in nanometers.

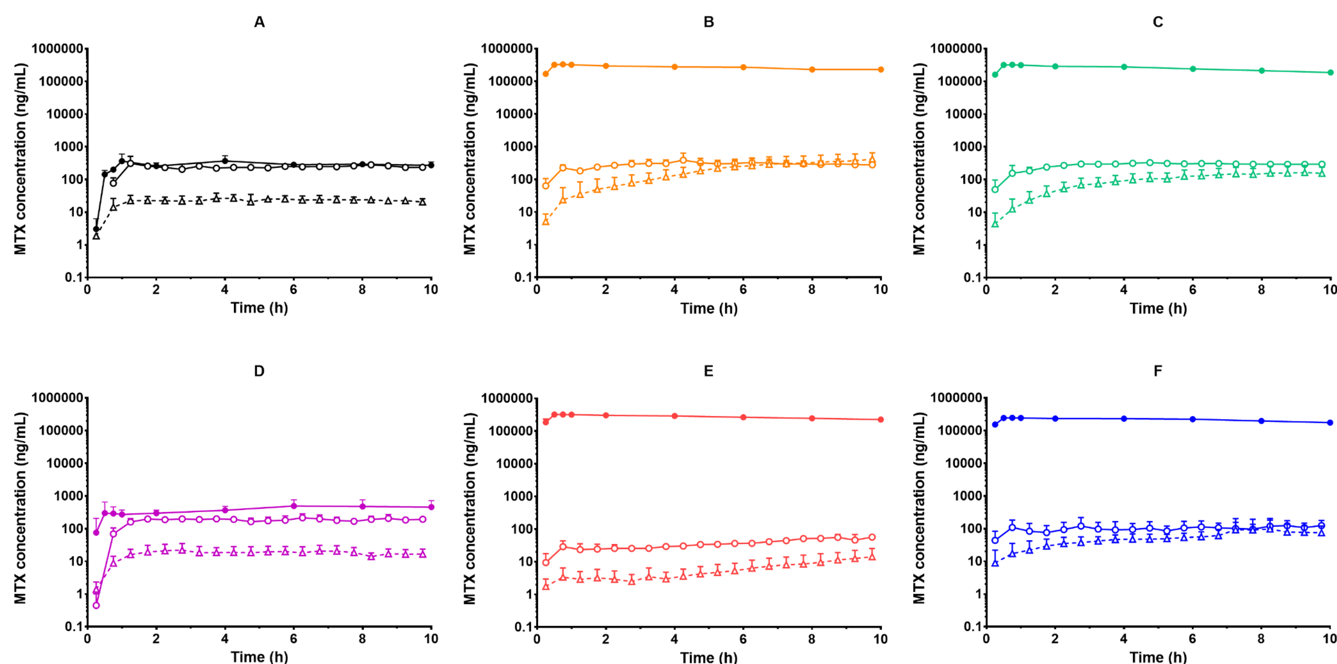

Fig. 2. Observed concentration-time profiles of unbound MTX in brain ISF (open triangles), unbound (open circles) and total MTX (filled circles) in plasma after i.v. administration of free MTX, liposomal MTX or free MTX + empty liposomes. (A) Free MTX (n = 4), (B) PEG-EYPC (n = 7), (C) GSH-PEG-EYPC (n = 7), (D) free MTX + empty GSH-HSPC-HSPC (n = 4), (E) PEG-HSPC (n = 7), (F) GSH-PEG-HSPC (n = 7). Data are presented as Mean ± SD.

Table 2
Estimated PK parameters of MTX in rats after the administration of free MTX, liposomal MTX or free MTX + empty liposomes. The calculation of CL, V_d and $t_{1/2}$ are based on total plasma concentrations. Data are presented as Mean ± SD.

Parameters	Administration groups					
	Free MTX	Free MTX + empty GSH-PEG-HSPC	PEG-HSPC	GSH-PEG-HSPC	PEG-EYPC	GSH-PEG-EYPC
CL (mL/h/kg)[§]	1240 ± 312	1030 ± 385	1.35 ± 0.28	1.90 ± 0.30	1.39 ± 0.28	1.71 ± 0.57
V_d (mL/kg)[§]	–	–	49.0 ± 7.3	58.7 ± 5.9	49.6 ± 5.2	53.7 ± 6.2
$t_{1/2}$ (h)[§]	–	–	27.0 ± 11.3	21.9 ± 4.5	25.5 ± 5.0	25.3 ± 13.7
f_u	0.76 ± 0.09	0.69 ± 0.07	–	–	–	–
$K_{p,uu}$	0.10 ± 0.03	0.09 ± 0.05	0.23 ± 0.17	0.82 ± 0.59[*,#]	1.5 ± 1.0[*]	0.53 ± 0.29[*]

[§] The residual areas for the liposomal formulations were 25–73% due to the very long half-lives.
[*] $p < 0.05$ indicates a significantly higher $K_{p,uu}$ compared with free MTX.
[#] $p < 0.05$ indicates a significantly higher $K_{p,uu}$ compared with PEG control liposomes.

Table 3
Steady-state total plasma, unbound plasma and unbound brain concentrations of MTX after administering free MTX, liposomal MTX or free MTX + empty liposomes, unbound-to-total and total-to-unbound plasma concentration ratios after administering liposomal MTX. Data are expressed as Mean ± SD.

Variable	MTX concentration (ng/mL)					
	Free MTX	Free MTX + empty GSH-PEG-HSPC	PEG-HSPC	GSH-PEG-HSPC	PEG-EYPC	GSH-PEG-EYPC
Total plasma	305 ± 80	394 ± 158	243000 ± 31300	192000 ± 21400	230000 ± 24500	204000 ± 23700
Unbound plasma	249 ± 32	213 ± 47	55.0 ± 9.2	111 ± 42	290 ± 87	292 ± 43
Unbound brain	23.5 ± 6.0	18.3 ± 8.2	11.8 ± 8.2	74.1 ± 43.7	360 ± 224	158 ± 95
$C_{u,ss,plasma}/C_{tot,ss,plasma}$			0.00024 ± 0.00005	0.00058 ± 0.00023	0.0013 ± 0.0004[&]	0.0015 ± 0.0003[&]
$C_{tot,ss,plasma}/C_{u,ss,plasma}$			4330 ± 826	2010 ± 819	855 ± 273	717 ± 162

[&] $p < 0.05$ indicates a significantly higher extent of MTX release in plasma compared with HSPC-based formulations.

were no significant differences between GSH and non-GSH formulations containing either HSPC or EYPC (Table 3).

The transport of free MTX at the BBB was very limited, reflected by a $K_{p,uu}$ of 0.10 ± 0.03 (Table 2, Fig. 3A, B and C). The co-administration of empty GSH-PEG-HSPC together with free MTX did not significantly influence MTX transport at the BBB compared with administering free MTX alone (Table 2, Fig. 3A and C).

Compared with administering free MTX, PEG-HSPC did not significantly affect brain uptake of MTX. PEG-EYPC on the other hand resulted in a significant improvement in uptake with a 15-fold increase in $K_{p,uu}$ to 1.5, although with a large variability ($p < 0.05$) (Table 2 and Fig. 3C). The addition of GSH on PEG-EYPC did not lead to further enhancement in brain delivery of MTX (Table 2 and Fig. 3B and C). Compared to PEG-HSPC, GSH-PEG-HSPC significantly increased the

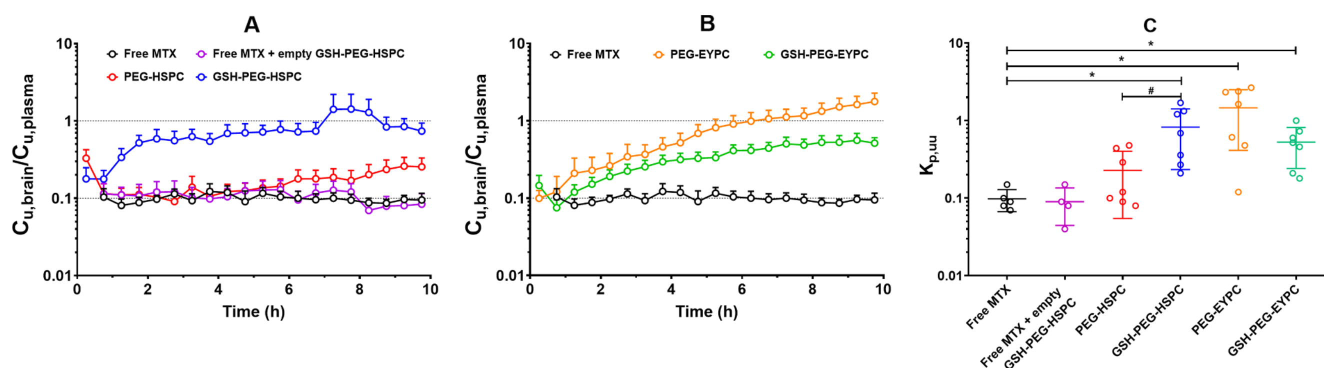

Fig. 3. (A) Unbound brain-to-plasma concentration ratios over time after 30 min i.v. infusion of free MTX, free MTX + empty PEG liposomes, PEG-HSPC or GSH-PEG-HSPC. Data are expressed as mean ± SEM, n = 4–7. (B) Unbound brain-to-plasma concentration ratios over time after 30 min i.v. infusion of free MTX, PEG-EYPC or GSH-PEG-EYPC. Data are presented as mean ± SEM, n = 4–7. (C) Steady-state unbound brain-to-plasma concentration ratio ($K_{p,uu}$) for the six groups. $^{*}p < 0.05$ indicates significantly higher $K_{p,uu}$ compared with free MTX, $^{\#}p < 0.05$ indicates significantly higher $K_{p,uu}$ compared with PEG control liposomes. n = 4–7. Comparisons were made based on the data between 8 and 10 h.

uptake of MTX into the brain with the $K_{p,uu}$ increasing to 0.82 ($p < 0.05$) (Table 2 and Fig. 3C). Also, the brain uptake was increased early after administration (Fig. 3A).

4. Discussion

Our study for the first time shows that the brain-targeting effect of GSH-PEG liposomal MTX is highly dependent on the liposomal formulation that is combined with GSH. While GSH-PEG-HSPC significantly improved the brain uptake of MTX by 4-fold compared to the non-targeted control PEG-HSPC, conjugation of GSH to PEG-EYPC did not result in a further increase in uptake. However, PEG-EYPC in itself showed significantly improved uptake compared with free MTX.

All four liposomal formulations gave similar total MTX levels in plasma that declined very slowly, independent of if they were based on HSPC or EYPC. This shows that all formulations were equally stable *in vivo*, confirming the results from the *in vitro* stability test, consistent with the results in our previous study [16]. The addition of GSH to either PEG-HSPC or PEG-EYPC did not make any difference in total plasma PK of MTX. This was in line with previous literature showing that GSH coating has no impact on the PK properties of the total drug in plasma compared to PEG control liposomes [10,11].

In order to fully map the behavior of nanoformulations *in vivo* and to more thoroughly understand possible improvement in brain uptake by the formulation, it is of importance to not only measure the total concentrations in plasma (mostly liposome-encapsulated and not available for the target) but also the concentrations of released, unbound drug (related to efficacy/toxicity). The use of microdialysis in blood successfully separated the released MTX from the drug remaining encapsulated, thus making it possible to continuously monitor the *in vivo* drug release from all formulations. In spite of similar total concentrations, PEG-EYPC released MTX faster in plasma than PEG-HSPC, as reflected by the significantly higher unbound-to-total plasma concentration ratio, and 5.3-fold higher unbound plasma concentration of MTX (Table 3). Our previous paper also showed faster MTX release from EYPC than HSPC based PEG liposomes [16]. However, the unbound-to-total plasma concentration ratios for both formulations were different between two studies (for PEG-HSPC: 0.00024 vs. 0.0007 and for PEG-EYPC: 0.0013 vs. 0.007 in the previous paper). The discrepancy in the extent of *in vivo* MTX release can likely be attributed to different PEGylation methods used in the two studies. Previously, 5% PEG was incorporated into both HSPC and EYPC based PEG liposomes using the pre-insertion method, which led to about half of the PEG being oriented outward from the membrane and the rest toward the inside of the liposomes [23]. In the current study, 4% PEG was post-inserted, leading to PEG being present mainly on the outer side of the liposomes, with around 4.5% PEG presented on the surface and 0.5% PEG facing inwards, including the 1% PEG pre-inserted. It can be speculated if different density of PEG on the inner and outer surfaces of the liposomes may influence the properties of liposome bilayers and consequently affect the *in vivo* release extent. The lack of significant difference in unbound-to-total concentration ratios in plasma between GSH-PEG and PEG control formulations suggests that GSH-coating did not influence the release properties of MTX in plasma regardless of the formulation used, consistent with the observations from a previous study [15].

Free MTX crossed the BBB to a very limited extent with a $K_{p,uu}$ being 0.1, confirming the findings from our previous study [16]. Also, similar to our previous results, PEG-HSPC did not significantly affect the transport of MTX at the BBB compared with administrating free MTX. However, unlike the 3-fold improvement in brain delivery of MTX relative to free MTX when PEG-EYPC liposomes were administered previously [16], we observed an even more substantial increase in $K_{p,uu}$ of MTX from 0.1 to 1.5 in this study from the PEG-EYPC group, in spite of a large variability. This finding is not consistent with a previous study showing that the increase in PEG density on nanoparticles decreased cellular uptake [24]. Currently, we do not have an explanation for this discrepancy. Despite this, the considerable increase of $K_{p,uu}$ from 0.1 to a value around unity was in itself an intriguing finding, indicating that the efflux transport of MTX might have been neutralized when delivering with PEG-EYPC liposomes.

GSH-PEG-EYPC did not result in a further increase in brain uptake compared to PEG-EYPC. Despite using a different payload (DAMGO, an opioid peptide), similar results were observed in a previous study where both GSH-PEG-EYPC and PEG-EYPC liposomes doubled brain uptake of DAMGO compared with administering free DAMGO without any significant difference in uptake between two formulations [15]. These results indicate that PEG-EYPC liposomes may not be a suitable formulation to be combined with GSH in order to achieve improved brain targeting delivery. On the contrary, PEG-EYPC liposomes seem to be able to improve brain drug delivery by themselves without the need of GSH. The most probable mechanism by which PEG-EYPC liposomes facilitated drug transport at the BBB was earlier interpreted as being a fusion of the liposomes with the endothelial luminal membrane [25]. When GSH was combined with PEG-EYPC, it can be speculated that GSH-mediated uptake may be dominated by the fusion process associated with PEG-EYPC, thus making the brain-targeting effect of GSH-PEG-EYPC not observable. Further mechanistic studies are required to elucidate the reason behind this phenomenon.

Unlike GSH-PEG-EYPC, GSH-PEG-HSPC significantly enhanced the transport of MTX at the BBB by 4-fold compared to its PEG control formulation and 8-fold compared to free MTX. In line with these results, previous studies have shown that GSH-PEG liposomes were superior to

plain PEG liposomes in delivering ribavirin, doxorubicin, methylprednisolone and carboxyfluorescein into the brain when HSPC was used as the main phospholipid [9–11,14,26]. Similar brain uptake was found regardless of whether free MTX was administered alone or together with empty GSH-PEG-HSPC liposomes. This observation excludes any possibility that GSH-PEG-HSPC liposomes themselves influence the BBB integrity or function. Moreover, as shown from $C_{u,brain}/C_{u,plasma}$ ratio with time (Fig. 3A and B), the EYPC based formulations gradually increased the brain uptake of MTX, while the GSH-PEG-HSPC formulation resulted in a much quicker increase in the uptake. Therefore, the increased brain delivery of MTX when using GSH-PEG-HSPC is indicative of a GSH-mediated targeting process, rather than non-specific interaction between liposomes and the BBB. The observation that PEG-HSPC liposomes did not at all influence the brain uptake compared with free MTX likely indicates that a fusion process like the one proposed for PEG-EYPC liposomes is not present for PEG-HSPC. Thus, GSH-mediated targeting is the most likely process for GSH-PEG-HSPC liposomes.

The therapeutic success of liposomal brain delivery is not solely dependent on the increase in $K_{p,uu}$ but is influenced by multiple factors. These factors include loading capacity, final drug concentration in liposome solution, volume allowed to be administered *in vivo*, release rate, improvement in brain delivery, attainable concentration at the target site to obtain clinical effects versus off-target site concentration to see side effects, and potency of the drug. Properties like high drug loading, appropriate release rate, large enough increase in $K_{p,uu}$ and high enough potency are favored and often needed to come together in order for liposomal delivery to be therapeutically successful at the CNS target. The half-life of the formulation is also of importance for therapeutic success. If administering free MTX with its short half-life and $K_{p,uu}$ of 0.1, in order to maintain an unbound brain concentration at therapeutic level, a constant infusion would have to be given to obtain a steady-state unbound plasma concentration of 4500 ng/mL. However, if GSH-PEG-HSPC or PEG-EYPC is administered, only a 30-min short infusion with a much lower steady-state unbound plasma concentration of 563 or 300 ng/mL, respectively, would be adequate. This is due to a prolonged half-life, sustainable drug release and greatly improved $K_{p,uu}$ when administering liposomal MTX. Therefore, when having similar central efficacy, delivery of MTX with certain liposomal formulations would have less risk of peripheral toxicity compared to giving free MTX.

Taking this study as an example, although GSH-PEG-HSPC substantially improved the $K_{p,uu}$ of MTX compared to PEG-HSPC and unformulated MTX, with an infusion volume of 0.94 mL, the resulting brain ISF concentration of MTX at steady-state was around 74 ng/mL (Table 3). This is much lower than the reported minimal effective concentration to kill tumor cells (450 ng/mL, or > 100 nM) [27]. Even if the maximal volume of 2 mL allowed to be administered to rats had been given, theoretically the brain ISF concentration would increase to 157 ng/mL, which would still not be sufficient to be therapeutically effective with a single administration. When the same MTX dose was administered in PEG-EYPC liposomes (infusion volume 0.77 mL), this resulted in a much higher brain ISF concentration of 360 ng/mL, which is a result of higher unbound-to-total plasma concentration ratio (0.0013 vs 0.00058) as well as higher $K_{p,uu}$ (1.5 vs 0.8) than the GSH-PEG-HSPC formulation. If 2 mL had been infused, the unbound brain concentration would have reached therapeutic level. From a safety window perspective, although PEG-EYPC has a better brain delivery, there may still be a concern for more peripheral side effects due to higher unbound plasma concentrations. Therefore, all these factors need to be taken into consideration when formulating a liposomal strategy for *in vivo* delivery to the brain.

5. Conclusions

Our study demonstrates that the brain-targeting effect of GSH-PEG liposomes, using MTX as the model drug, relies highly on the liposomal formulation that is combined with GSH. Compared to the PEG control formulations, GSH-PEG-HSPC increased brain delivery of MTX 4-fold (8-fold compared to free MTX), while the addition of GSH on PEG-EYPC did not result in a further enhancement of brain uptake. On the other hand, PEG-EYPC was in itself improving uptake 15-fold (5-fold with GSH-PEG-EYPC), yet with higher unbound plasma concentrations. Since co-administration of empty GSH-PEG-HSPC liposomes with free MTX did not affect uptake, the improved brain delivery was not caused by liposomes themselves influencing BBB integrity or function, but more likely a consequence of a GSH-mediated process. This work deepens the understanding of how liposomal formulations could influence the outcomes of brain-targeted delivery of GSH-PEG liposomes, highlighting the central role of formulation optimization when developing this promising brain delivery technology to treat CNS diseases. Since a brain-specific targeted delivery of drugs (as seen by the GSH-PEG liposomes using HSPC) is generally considered to be preferred for overall successful clinical drug development over a non-specific tissue delivery (as seen by the PEG liposomes using EYPC), we conclude that finding the optimal dosing schedule of the GSH-PEG liposomes using HSPC is the preferred way forward for MTX delivery to the CNS.

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References

[1] N.J. Abbott, Blood-brain barrier structure and function and the challenges for CNS drug delivery, J. Inherit. Metab. Dis. 36 (2013) 437–449.
[2] N. Strazielle, J.F. Ghersi-Egea, Physiology of blood-brain interfaces in relation to brain disposition of small compounds and macromolecules, Mol. Pharm. 10 (2013) 1473–1491.
[3] R. Karim, C. Palazzo, B. Evrard, G. Piel, Nanocarriers for the treatment of glioblastoma multiforme: Current state-of-the-art, J. Control. Release 227 (2016) 23–37.
[4] J. Rip, Liposome technologies and drug delivery to the CNS, Drug Discov. Today Technol. 20 (2016) 53–58.
[5] P.J. Gaillard, C.C. Visser, M. Boer, C.C. Appeldoorn, J. Rip, Blood-to-brain drug delivery using nanocarriers, in: M. Hammarlund-Udenaes, E.C. de Lange, R.G. Thorne (Eds.), Drug Delivery to the Brain, Springer, New York, 2014, pp. 433–454.
[6] R. Kannan, J.F. Kuhlenkamp, E. Jeandidier, H. Trinh, M. Ookhtens, N. Kaplowitz, Evidence for carrier-mediated transport of glutathione across the blood-brain barrier in the rat, J. Clin. Invest. 85 (1990) 2009–2013.
[7] B.V. Zlokovic, J.B. Mackic, J.G. McComb, M.H. Weiss, N. Kaplowitz, R. Kannan, Evidence for transcapillary transport of reduced glutathione in vascular perfused guinea-pig brain, Biochem. Biophys. Res. Commun. 201 (1994) 402–408.
[8] R. Kannan, R. Chakrabarti, D. Tang, K.J. Kim, N. Kaplowitz, GSH transport in human cerebrovascular endothelial cells and human astrocytes: evidence for luminal localization of Na+-dependent GSH transport in HCEC, Brain Res. 852 (2000) 374–382.
[9] P.J. Gaillard, C.C. Appeldoorn, J. Rip, R. Dorland, S.M. van der Pol, G. Kooij, H.E. de Vries, A. Reijerkerk, Enhanced brain delivery of liposomal methylprednisolone improved therapeutic efficacy in a model of neuroinflammation, J. Control. Release 164 (2012) 364–369.
[10] J. Rip, L.D. Chen, R. Hartman, A. van den Heuvel, A. Reijerkerk, J. van Kregten, B. van der Boom, C. Appeldoorn, M. de Boer, D. Maussang, E.C.M. de Lange, P.J. Gaillard, Glutathione PEGylated liposomes: pharmacokinetics and delivery of cargo across the blood-brain barrier in rats, J. Drug Target. 22 (2014) 460–467.
[11] P.J. Gaillard, C.C. Appeldoorn, R. Dorland, J. van Kregten, F. Manca, D.J. Vugts, B. Windhorst, G.A. van Dongen, H.E. de Vries, D. Maussang, O. van Tellingen, Pharmacokinetics, brain delivery, and efficacy in brain tumor-bearing mice of glutathione pegylated liposomal doxorubicin (2B3-101), PLoS One 9 (2014) e82331.
[12] A. Lindqvist, J. Rip, P.J. Gaillard, S. Bjorkman, M. Hammarlund-Udenaes, Enhanced brain delivery of the opioid peptide DAMGO in glutathione pegylated liposomes: a microdialysis study, Mol. Pharm. 10 (2013) 1533–1541.
[13] M. Rotman, M.M. Welling, A. Bunschoten, M.E. de Backer, J. Rip, R.J.A. Nabuurs, P.J. Gaillard, M.A. van Buchem, S.M. van der Maarel, L. van der Weerd, Enhanced glutathione PEGylated liposomal brain delivery of an anti-amyloid single domain antibody fragment in a mouse model for Alzheimer's disease, J. Control. Release

203 (2015) 40–50.
[14] C.C.M.J. Rip, F.M. Appeldoorn, R. Manca, J.M.R.V.K. Dorland, P.J. Gaillard, Receptor-mediated delivery of drugs across the blood–brain barrier, in: Front. Pharmacol. Conference Abstract (2010): Pharmacology and Toxicology of the Blood–brain Barrier: State of the Art, Needs for Future Research and Expected Benefits for the EU, 2010, https://doi.org/10.3389/conf.fphar.2010.02.00025.
[15] A. Lindqvist, J. Rip, J. van Kregten, P.J. Gaillard, M. Hammarlund-Udenaes, In vivo functional evaluation of increased brain delivery of the opioid peptide DAMGO by glutathione-PEGylated liposomes, Pharm. Res. 33 (2016) 177–185.
[16] Y. Hu, J. Rip, P.J. Gaillard, E.C.M. de Lange, M. Hammarlund-Udenaes, The impact of liposomal formulations on the release and brain delivery of methotrexate: an in vivo microdialysis study, J. Pharm. Sci. 106 (2017) 2606–2613.
[17] M.R. Bouw, M. Hammarlund-Udenaes, Methodological aspects of the use of a calibrator in in vivo microdialysis-further development of the retrodialysis method, Pharm. Res. 15 (1998) 1673–1679.
[18] A. Gupta, P. Chatelain, R. Massingham, E.N. Jonsson, M. Hammarlund-Udenaes, Brain distribution of cetirizine enantiomers: comparison of three different tissue-to-plasma partition coefficients: K(p), K(p, u), and K(p, uu), Drug Metab. Dispos. 34 (2006) 318–323.
[19] M. Hammarlund-Udenaes, M. Friden, S. Syvanen, A. Gupta, On the rate and extent of drug delivery to the brain, Pharm. Res. 25 (2008) 1737–1750.
[20] R. Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2018 https://www.R-project.org/.
[21] T. Hothorn, F. Bretz, P. Westfall, Simultaneous inference in general parametric models, Biom J 50 (2008) 346–363.
[22] F. Bretz, T. Hothrn, P. Westfall, Mutiple Comparisons using R, CRC Press, Boca Raton, 2010.
[23] S.H. Lee, Y. Sato, M. Hyodo, H. Harashima, Topology of surface ligands on liposomes: characterization based on the terms, incorporation ratio, surface anchor density, and reaction yield, Biol. Pharm. Bull. 39 (2016) 1983–1994.
[24] X. Han, Z. Li, J. Sun, C. Luo, L. Li, Y. Liu, Y. Du, S. Qiu, X. Ai, C. Wu, H. Lian, Z. He, Stealth CD44-targeted hyaluronic acid supramolecular nanoassemblies for doxorubicin delivery: probing the effect of uncovalent pegylation degree on cellular uptake and blood long circulation, J. Control. Release 197 (2015) 29–40.
[25] A. Lindqvist, M. Friden, M. Hammarlund-Udenaes, Pharmacokinetic considerations of nanodelivery to the brain: Using modeling and simulations to predict the outcome of liposomal formulations, Eur. J. Pharm. Sci. 92 (2016) 173–182.
[26] T. Birngruber, R. Raml, W. Gladdines, C. Gatschelhofer, E. Gander, A. Ghosh, T. Kroath, P.J. Gaillard, T.R. Pieber, F. Sinner, Enhanced doxorubicin delivery to the brain administered through glutathione PEGylated liposomal doxorubicin (2B3-101) as compared with generic Caelyx, ((R))/Doxil((R))–a cerebral open flow microperfusion pilot study, J. Pharm. Sci. 103 (2014) 1945–1948.
[27] W.M. Hryniuk, J.R. Bertino, Treatment of leukemia with large doses of methotrexate and folinic acid: clinical-biochemical correlates, J. Clin. Invest. 48 (1969) 2140–2155.