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Cerebral Hypoperfusion-assisted Intraarterial Deposition of Liposomes in Normal and Glioma-bearing Rats

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Abstract

Background—Optimizing liposomal vehicles for targeted delivery to the brain has important implications for the treatment of brain tumors. The promise of efficient, brain-specific delivery of chemotherapeutic compounds via liposomal vehicles has yet to be achieved in clinical practice. Intra-arterial injection of specially designed liposomes may facilitate efficient delivery to the brain and to gliomas.

Objective—To test the hypothesis that cationic liposomes may be effectively delivered to both normal and glioma-bearing brain tissue utilizing a strategy of intra-arterial injection during transient cerebral hypoperfusion.

Methods—Cationic, anionic, and neutral liposomes were separately injected via the internal carotid artery of healthy rats during transient cerebral hypoperfusion. Rats bearing C6 gliomas were similarly injected with cationic liposomes. Liposomes were loaded with DiI_{C18}(5) dye whose concentrations can be measured by light absorbance and fluorescence methods.

Results—After intra-arterial injection, a robust uptake of cationic as compared to anionic and neutral liposomes into brain parenchyma was observed by diffuse reflectance spectroscopy. Post mortem multispectral fluorescence imaging revealed that liposomal cationic charge was associated

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with more efficient delivery to the brain. Cationic liposomes were also readily observed within glioma tissue after intra-arterial injection. However, over time, cationic liposomes were retained longer and at higher concentrations in the surrounding, peri-tumoral brain than in the tumor core.

Conclusion—This study demonstrates the feasibility of cationic liposome delivery to brain and glioma tissue after intra-arterial injection. Highly cationic liposomes directly delivered to the brain via an intracarotid route may represent an effective method for delivering anti-glioma agents.

Keywords

blood-brain barrier; chemotherapy; drug delivery; glioma; intraarterial

Introduction

Liposomal vehicles have been proposed as an effective means for delivering drugs to treat malignancy. However, traditional intravenous (IV) liposome delivery is limited by non-specific uptake by circulating white blood cells and by organs such as the liver and spleen.^{1, 2} Non-target uptake can, in part, be minimized by intra-arterial (IA) delivery.³⁻⁷ Recent reports suggest a significant brain uptake of magnetically-targeted cationic liposomes after IA injection.⁸ Additionally, computational models and experimental data suggest that IA delivery to the brain can be greatly increased if it is done during transient cerebral hypoperfusion (TCH).^{4, 9-12} Such injections achieve higher arterial blood concentration, have longer cerebral transit time, and avoid the problem of streaming.^{13, 14} Reducing blood flow could also decrease shear stress that dislodges particles adherent to the vascular endothelium.^{15, 16}

In this study we sought to harness the benefits of both intracarotid injection and cerebral flow reduction to test the effects of liposome electrostatic charge on targeted delivery to normal and glioma-bearing brain. We show that cationic liposomes can be effectively delivered in these scenarios, and demonstrate the kinetics and dynamics of this targeted delivery technique.

Methods

Materials

Dimyristoylphosphatidylglycerol (DMPG), dimyristoylphosphatidylcholine (DMPC), and dioleoyl-trimethylammonium-propane (DOTAP) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol) was obtained from Sigma-Aldrich (St. Louis, MO). The non-exchangeable dialkyl carbocyanine membrane label DiI_{C18}(5) (DiD) was obtained from Invitrogen (Carlsbad, CA).

Liposome Preparation

Liposomes were prepared by hydration of dried lipid films with NaCl/TRIS buffer followed by sequential extrusion through polycarbonate filters to a final diameter of 80 nm.¹² Cationic liposomes were composed of DMPC:DOTAP:Chol:DiD (2.75:x:4.5:1.1 mole % each), where 'x' represents DOTAP in amounts corresponding to 5, 25, and 50 mole % of the total dialkyl + diacyl lipids. Charge-neutral liposomes were composed of

DMPC:Chol:DiD (5.5:4.5:1.1 mole %)¹⁷. Anionic liposomes were composed of DMPG:Chol:DiD (5.5:4.5:1.1 mole %). Prior to injection, liposomes were diluted to 10 mM in 25 mM TRIS/150 mM NaCl pH 7.2 buffer so as to deliver comparable doses of the DiD liposome tracer dye.

Animal Surgery and Monitoring

Rats were cared for in accordance with the Columbia University Institutional Animal Care and Use Committee (IACUC). Male Sprague Dawley rats were utilized for all animal experiments. Details of anesthetic and surgical preparation have previously been described.¹² Briefly, anesthesia is maintained with isoflurane, ketamine, and propofol as needed. The femoral artery is cannulated for blood pressure monitoring. The internal carotid artery is isolated and cannulated. The rat is then placed on a stereotactic frame and the skull is exposed through a midline incision. The right parietal bone is carefully thinned to the inner table such that cortical vessels can clearly be seen. The optical pharmacokinetic (OP) technique uses diffuse reflectance spectroscopy for which the probe is placed over the thinned skull such that the probe face is in gentle contact with the inner table and orthogonal to the surface of the brain.¹⁸ The skull region anterior to bregma is similarly prepared and a laser Doppler probe is placed to monitor cerebral blood flow. Additional parameters monitored during experiments include: electrocardiography, mean arterial pressure, pulse oxygen saturation, skin blood flow, inspired and expired gas composition (oxygen, carbon dioxide, isoflurane), and rectal and esophageal temperature.

Glioma Preparation

C6 glioma cells (ATCC) were sub-cultured to confluence and 1×10^6 fresh tumor cells were stereotactically injected in the rat brain at 3 mm lateral, 2 mm posterior, and 3.5 mm deep to bregma.¹⁹ After implantation the 1 mm burr hole was sealed with wax and irrigated with hydrogen peroxide. The incision was closed in a single layer and post-operative pain medication (Buprenorphine) and antibiotics (Baytril) were administered. Experiments were performed between 9 to 12 days-post-injection.

Intracarotid Injection

Liposomes were injected as a series of 15 micro-boluses of approximately 65 μ l (total 1 ml) while transiently decreasing the cerebral blood flow. The boluses were injected using a pneumatically driven pressure injector (Picospritzer III, Parker Hannifin, Pine Brook, NJ) controlled by a signal generator (33220A, Agilent Technologies Inc., Santa Clara, CA). This system allowed manipulation of bolus volume and injection duration through pulse-width modulation of the input square wave. In order to maximize drug exposure and evenness of distribution, the volume of the pulse was chosen such that the drug bolus saturates the arterial and capillary vessels but only minimally displaces blood on the venous end. In order to obtain this approximate volume, preliminary studies were conducted with saline using optical coherence tomography (OCT).²⁰ The OMAG (optical micro-angiography) method was used to delineate artery and veins in a cross-sectional B-scan. IA saline bolus volumes tested were from 20 to 100 μ l. The optimal pulse volume was selected to be approximately 60-70 μ l.

Transient Cerebral Hypoperfusion

Cerebral hypoperfusion was achieved by contralateral carotid artery occlusion and administration of intravenous adenosine (2-5 mg) and esmolol (2-3 mg). In rats this produces transient hypotension lasting on the order of seconds. This duration may be greatly increased by flushing with cold saline (4°C). Transient bradycardia and hypotension are also seen under these circumstances and may last 2-5 minutes.¹²

Diffuse Reflectance Spectroscopy

Diffuse reflectance spectroscopy was used to provide the real-time OP measurements and employed custom-built spectrophotometers (Optimum Technology Inc. Boston MA). A xenon arc lamp generates white light (450-850 nm) that is transmitted to the tissue by a fiber optic cable and tissue back-scattered light is transmitted to the instrument via a collection fiber. The wavelength-dependent reflected light intensity is read by a spectrometer and transferred into Matlab for further analysis. A modified Beers law model was used to determine the tissue concentrations of DilC₁₈ (5), oxy- and deoxyhemoglobin for each acquired spectrum. In total, 612 spectra were obtained for each injection over a period of approximately 30 minutes to sample the liposome concentration-time curves.^{18, 21-26}

Multispectral Imaging

Fluorescence images of post mortem tissues were acquired by a custom built spectral imaging system.²⁷ Excitation of the specimen was done by a white light LED (MWWHL3, Thorlabs, Newton, NJ) equipped with a liquid crystal tunable filter (VARISPC, Perkin Elmer, Waltham, MA) configured to 640 ± 10 nm. Fluorescence signals were acquired by a CCD camera (Prosilica, Allied Vision technology, GmbH, Stadtroda, Germany) positioned directly above the target. A 650 nm dichroic long-pass filter was used to couple the optical paths of the light source and imager and allow for even illumination over the field of view (Edmund Optics, Barrington, NJ). Fluorescence images were filtered by a 680 ± 10 nm band-pass filter mounted onto the lens of the camera. A microcontroller was used to synchronize camera frame acquisition with LED strobe settings. Image acquisition was controlled by a custom GUI written in C. Fluorescence images were imported into Matlab (Mathworks, Natick, MA) for image processing. Samples of the injected liposome formulation were drawn into capillary tubes and imaged to provide standards for normalizing tissue staining intensity for each experiment.

Postmortem tissue drug concentrations

Multispectral imaging was used to first identify an area of minimal fluorescence of the liposomes in post mortem brain tissue. Once the site was identified, the OP probe was used to determine an in-animal baseline (unstained) spectrum for diffuse reflectance spectroscopy based OP measurements. OP measurements were then taken from anterior (ACA), middle (MCA), and posterior cerebral artery (PCA) distribution sites on both the ipsi- and contralateral hemispheres. Reflectance spectra were normalized at the oxy- and deoxyhemoglobin isosbestic point, 800 nm, to account for changes in total hemoglobin between measurements. DilC₁₈(5) concentrations were determined and compared for both

hemispheres and with respect to the ipsilateral MCA distribution, the primary region of intracarotid drug injection.

Statistical analysis

Statistical analysis was done using the Stat View 5.2 (SAS Institute, Cary NC). Data were analyzed using ANOVA and factorial ANOVA. Statistical significance was set at $p < 0.05$.

Results

Effect of surface charge on liposomal delivery to brain

The differential efficacy of delivering cationic, anionic, and neutral liposomes to normal brain utilizing TCH-assisted intracarotid delivery was initially assessed using diffuse reflectance spectroscopy for real-time OP measurement. Each group of five animals was compared for differences in both measured and calculated parameters of tissue concentration (Table 1A). Cationic liposomes were observed to accumulate at the highest concentrations in the brain. Both peak and end tissue concentrations were greatest for cationic liposomes as compared to neutral and anionic liposomes. Neutral liposomes had slightly higher end-concentrations and retention ratios than anionic liposomes. The measured hemodynamic parameters also did not vary among animals receiving the different liposome groups (Table 1B).

Post mortem multispectral imaging of brain tissue specimens from each group was used to assess the distribution pattern of the liposomes and to determine the relative tissue concentrations at the site of the real-time OP measurements. These studies also demonstrated the significant retention of cationic liposomes as compared to anionic and neutral liposomes in the ipsilateral cerebral hemisphere (Fig. 1). Similarly, diffuse reflectance spectroscopy showed that liposome concentrations in the ipsilateral cerebral hemisphere were greater than in the contralateral hemisphere. Both techniques corroborate that there is significantly greater brain retention of cationic liposomes as compared to anionic and neutral liposomes (Fig. 2).

Effect of cationic charge density on liposome delivery to brain

The effect of cationic charge density on delivery efficiency was assessed by analyzing brain concentration for 5%, 25%, and 50% DOTAP-containing liposomes after intracarotid delivery. Each group contained six animals. The tissue concentrations and pharmacokinetic parameters of the 5% DOTAP-containing group were significantly lower than was seen in the 25% and 50% DOTAP-containing groups (Table 2A). Although 50% DOTAP liposomes achieved the highest peak concentration, it also demonstrated the most rapid clearance, such that the end concentration was not different from that of 25% DOTAP liposomes (Table 2A, Fig. 3). Interestingly, 25% as compared to 50% DOTAP-containing liposomes seemed to be better retained in postmortem specimens, but the difference was only significant in the contralateral middle (MCA) and posterior cerebral artery (PCA) distributions (Fig. 4). Similar to the initial experiments with 50% DOTAP liposomes, there was no significant difference in the measured hemodynamic parameters during delivery of the different cationic liposomes (Table 2B).

Distribution of cationic liposomes in C6 glioma-bearing brains

A total of 8 rats were injected with C6 glioma cells and subsequently underwent TCH-assisted intracarotid liposome delivery. Confocal fluorescence microscopy confirmed that liposomes can be delivered effectively to the glioma. Liposomes were found to penetrate the tumor mass but were retained in higher concentrations and for a longer time periods in the peri-tumor tissue (Fig. 5 and 6).

Discussion

The results of this study demonstrate several important features of IA liposomal delivery to the brain and to gliomas. First, the value of using positive surface charge to enhance liposomal delivery during IA flow arrest is confirmed. Second, this study shows that maximum surface charge is not required for effective IA delivery to the CNS, which could be important to minimize potential adverse effects of cationic liposomes. Third, it demonstrates that cationic liposomes may accumulate and redistribute within the brain and have parenchymal concentrations that decline over time. In comparison, anionic and neutral liposomes achieve much lower brain concentrations and are minimally retained. Fourth, this study demonstrates that cationic liposomes are able to penetrate the glioma core, possibly with greater concentrations and longer retention times in the tumor periphery. Finally, this study demonstrates that an array of optical methods, including minimally invasive approaches, can now be employed to assess nanoparticle delivery, which could assist in future clinical drug development.

Intra-arterial drug delivery

Since the 1950s, IA drug delivery has been evaluated for the treatment of malignant brain tumors.^{4, 28-30} However, despite intensive efforts, IA drug delivery has yet to make a significant therapeutic impact in the treatment of brain cancers—a failure it shares with many other malignant brain tumor treatments. This is somewhat remarkable given that for cancers of other organs such as liver, breast, eye, and even extra-cranial tumors of the head and neck, IA chemotherapy is fairly effective.³¹⁻³⁴ It is a frontline treatment for retinoblastoma and for advanced liver metastasis. In general, IA drugs are most effective in three specific situations: 1. when there is low regional blood flow, 2. when there is high regional drug extraction, and 3. when the drug is rapidly cleared from the systemic circulation.³⁰ In addition, more consistent tissue drug concentrations are achieved by pulsed or bolus dosing.^{30, 35, 36} Therefore, our approach to IA delivery to the brain uses transient blood flow reduction with concurrent intracarotid bolus injection.¹⁰⁻¹² IA injection during transient cerebral hypoperfusion generates high cerebral arterial drug concentrations, increases drug transit time through brain tissue, and minimizes drug binding to blood proteins and cell components. In the case of nanoparticle delivery, flow arrest decreases shear stress and thereby increases the probability of nanoparticle adhesion to the target site.^{15, 16}

Hypoperfusion assisted delivery

The idea of reducing blood flow to improve delivery has been used clinically while treating cancers in several organs. In the cases of liver and breast cancer, stop-flow drug delivery can

increase tumor site concentrations up to 10-fold.³¹ In the case of retinoblastoma, Yamane et al. reduced non-target downstream flow by supra-ophthalmic balloon occlusion for better targeting chemotherapy. They used flow occlusion during 568 clinical procedures in 187 children as young as 35 days, with no adverse effects.³⁷ Recently, Riina et al. have used vertebral artery balloon occlusion to assist in the IA delivery of chemotherapy to gliomas.³⁸ In the course of treatment, the artery was occluded for 3 minutes, and then reperused for 2 minutes, for several cycles. The underlying rationale was to improve Bevacizumab targeting to the tumor tissue. Although some have voiced reasonable concerns regarding the risks of neurological complications after hypoperfusion-assisted drug delivery, those risks have to be assessed against the inexorable advance and fatal outcome expected from malignant gliomas.

Liposome charge characteristics

This study underscores the advantage of deploying cationic liposomes over anionic and neutral liposomes for improving IA drug delivery. As previously hypothesized by Zhao et al., negative charge may shield opsonins and enhance binding to the negatively charged endothelium, which may facilitate penetration across the blood-brain barrier⁸. Interestingly, it was observed that cerebral retention of cationic liposomes after intracarotid injection is not directly related to the amount of positive surface charge. Our results suggest that the optimal surface charge that results in the most favorable amount of liposome retention can be achieved with molar fractions of cationic lipid that are quite modest. This finding has important implications for both drug loading into liposomal vehicles and for efficient brain delivery. Many anthracycline chemotherapeutic drugs, such as daunorubicin, doxorubicin, and mitoxantrone, carry a positive charge that can pose technical hurdles when loading these drugs into cationic liposomes. Our demonstration that surface charge can be reduced to 25 mole% of the membrane lipids improves the feasibility of producing cationic liposomal formulations of these drugs for delivery to brain.

Liposomal delivery to brain tumor

In C6 glioma-bearing rats, we observed not only that liposomes could be delivered to the tumor center after intracarotid injection, but also that they persisted longer and at higher concentrations within the peri-tumor brain tissue. One explanation for this finding may be that neovascularization and leaky capillaries at the tumor core results in a net outward flux of liposomes toward the periphery. Additionally, the disrupted blood-tumor barrier may result in increased liposome removal due to back diffusion into the circulating blood. High-resolution confocal microscopy showed a uniform distribution of the liposome membrane marker dye, suggesting that liposomes can redistribute after extravasation and penetrate into regions containing glioma cells. Detailed analyses of the release of drug cargo from the cationic liposomes that penetrate into the tumor await future studies. Using current technology, combining confocal microscopy and real-time spectroscopy, it is possible to reveal cell- and tissue- level drug kinetics and drug metabolism for compounds such as mitoxantrone and doxorubicin.

Conclusion

This study demonstrates the capability for delivering cationic liposomes by hypoperfusion-assisted IA delivery to the rat brain. It reveals that these liposomes may be retained in both healthy and glioma-bearing brain tissue after IA delivery for several hours. Maximum liposome surface charge is not necessary for optimal tissue retention of liposomes. IA delivery of cationic liposomes may provide a valuable means of delivering chemotherapeutic drugs, radio-sensitizers, and short half-life beta emitters for treating malignant brain tumors.

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Figure 1. Representative brain liposome concentration-time curves and multispectral fluorescence images

The concentration-time curves generated by diffuse reflectance spectroscopy indicate preferential brain uptake and retention of cationic liposomes as compared to anionic and neutral liposome after intraarterial delivery (**A**). Post-mortem brain sample with corresponding multispectral fluorescence image shows high retention of cationic liposomes in the ipsilateral cerebral hemisphere (**B**). In comparison, weak signal on multispectral fluorescence images after anionic (**C**) and neutral liposome (**D**) delivery indicates poor brain targeting of these liposomes after intraarterial delivery.

Figure. 2. Charge-specific regional distribution of liposomes

Diffuse reflectance spectroscopy of post mortem samples demonstrates the regional deposition of liposomes with different surface charges in specified arterial distributions. Significantly increased uptake of cationic liposomes is observed across all arterial distributions of the ipsilateral hemisphere as compared with deposition of anionic and neutral liposomes.

Figure. 3. Representative concentration-time curves and post mortem multispectral fluorescence images of cationic liposomes

50% DOTAP liposomes achieved the highest peak concentration but also had the most rapid clearance. The end tissue concentration of 25% DOTAP liposomes was the same as that observed with 50% DOTAP liposomes (**A**). Post-mortem brain samples with corresponding multispectral fluorescence images show relatively increased retention of 50% (**B**) and 25% (**C**) DOTAP liposomes as compared to 5% (**D**) DOTAP liposomes which had minimal fluorescence signal.

Figure. 4. Regional deposition of cationic liposomes of differing charge density

Quantitative assessment of post-mortem brain samples by diffuse reflectance spectroscopy indicates that the lowest cationic charge density liposomes (5% DOTAP) tested had the least retention across all arterial distributions. A trend toward increased retention of 25% as compared to 50% DOTAP liposomes was also seen across all arterial distributions suggesting that optimal rather than maximal cationic charge loading is necessary for efficient intrarterial delivery.

Figure. 5. Distribution of cationic liposomes in glioma-bearing brain

Gross section through a C6 glioma-bearing brain is shown. Labeled (B-E) locations on the gross specimen correspond to the regions shown by confocal microscopy (A). Confocal micrographs of brain sections contralateral to tumor (B), ipsilateral but remote from tumor (C), within the tumor (D), and in the peri-tumoral tissue (E) are shown. DAPI stained cell nuclei (blue) and the fluorescent liposome membrane label DiD (red) indicate liposome deposition relative to cells. Thus, high levels of liposome retention are achieved both within and in the immediate periphery of the tumor. Tu - tumor

Figure. 6. Time course of cationic liposome retention in glioma-bearing brain

Gross brain section through tumor (**A-C**) with corresponding multispectral fluorescence images (**D-F**) and H&E stained sections (**G-I**) at 30 minutes (top row), 2 hours (middle row), and 4 hours (bottom row) after intraarterial liposome delivery are shown. High levels of fluorescence, indicative of liposome localization, are seen both within and surrounding the tumor at 30 minutes (**D**) and after 2 hours (**E**). The highest levels of fluorescence appear to be peritumoral. By 4 hours after intraarterial delivery much lower levels of fluorescence are seen within the tumor (**F**) although discrete areas of high intensity can be seen in regions surrounding the tumor. Tu - tumor

Table 1A
Effect of liposomal surface charge on intracarotid delivery to brain

Tissue concentrations:	Anionic (n=5)	Cationic (n=5)	Neutral (n=5)
<i>Peak concentration (μM)</i>	0.26 $\pm$ 0.12	0.42 $\pm$ 0.07	0.18 $\pm$ 0.04 ^a
<i>AUC injection ($\mu\text{M}\cdot\text{s}$)</i>	5.67 $\pm$ 2.90	8.04 $\pm$ 0.64	3.61 $\pm$ 1.90 ^a
<i>AUC clearance ($\mu\text{M}\cdot\text{s}$)</i>	42 $\pm$ 33 ^a	335 $\pm$ 36	53 $\pm$ 41 ^a
<i>AUC total ($\mu\text{M}\cdot\text{s}$)</i>	48 $\pm$ 34 ^a	343 $\pm$ 36	56 $\pm$ 43 _a
<i>End Concentration (μM)</i>	0.03 $\pm$ 0.03 ^b	0.16 $\pm$ 0.03	0.05 $\pm$ 0.3 ^a
<i>Retention ratio (end/peak conc.)</i>	0.10 $\pm$ 0.11 ^{ab}	0.39 $\pm$ 0.04	0.25 $\pm$ 0.10 ^a

^aSignificant difference from cationic liposomes

^band neutral liposome

AUC: area under curve

Table 1B
Effect of liposomal surface charge on hemodynamic parameters during transient cerebral hypoperfusion

	Base	Peak	5 min	End
<i>Anionic (n=5)</i>				
MAP (mm Hg)	65±9	15±3	60±13	72±12
HR (bpm)	286±42	73±21	283±50	268±46
% CBF	100±0	9±3	104±13	96±21
<i>Cationic (n=5)</i>				
MAP (mm Hg)	65±8	18±3	57±7	64±7
HR (bpm)	225±42	42±49	248±41	291±14
% CBF	100±0	17±7	81±8	82±21
<i>Neutral (n=5)</i>				
MAP (mm Hg)	79±18	28±19	70±26	722±26
HR (bpm)	262±40	129±84	268±46	301±27
% CBF	100±0	13±7	114±22	96±22

MAP: mean arterial pressure, *HR*: heart rate; % *CBF*: % change in cerebral blood flow

Table 2A
Effect of cationic charge intensity on IA-TCH assisted delivery of liposomes

Tissue concentrations:	5% (n=6)	25% (n=6)	50%(n=5) (n=6)
<i>Peak concentration (μM)</i>	0.2 $\pm$ 0.07	0.35 $\pm$ 0.08*	0.42 $\pm$ 0.11*
<i>AUC injection ($\mu\text{M}\cdot\text{s}$)</i>	4.06 $\pm$ 1.71	8.27 $\pm$ 2.71	9.11 $\pm$ 5.26
<i>AUC clearance ($\mu\text{M}\cdot\text{s}$)</i>	35 $\pm$ 27	295 $\pm$ 109*	317 $\pm$ 66*
<i>AUC total ($\mu\text{M}\cdot\text{s}$)</i>	39 $\pm$ 28	303 $\pm$ 110*	326 $\pm$ 69*
<i>End Concentration (μM)</i>	0.04 $\pm$ 0.03	0.17 $\pm$ 0.06*	0.15 $\pm$ 0.06*
<i>Retention ratio (end/peak conc.)</i>	0.2 $\pm$ 0.07	0.35 $\pm$ 0.08*	0.42 $\pm$ 0.11*

* Significant difference from 5% DOTAP liposomes

Table 2B
Effect of cationic charge intensity on hemodynamic parameters during transient cerebral hypoperfusion

	Base	Peak	5 min	End
<i>5% DOTAP (n=6)</i>				
MAP (mm Hg)	66±13	21±12	54±9	65±15
HR (bpm)	272±32	73±57	274±41	293±13
% CBF	100±0	14±5	82±48	101±25
<i>25% DOTAP (n=6)</i>				
MAP (mm Hg)	60±12	23±9	50±8	58±12
HR (bpm)	304±20	73±59	293±36	312±24
% CBF	100±0	18±9	95±54	125±28
<i>50% DOTAP (n=6)</i>				
MAP (mm Hg)	77±14	18±2	49±14	70±17
HR (bpm)	277±35	43±38	245±32	300±18
% CBF	100±0	21±18	75±16	87±34

MAP: mean arterial pressure, HR: heart rate; % CBF: % change in cerebral blood flow