Significant Transport of Doxorubicin into the Brain with Polysorbate 80-Coated Nanoparticles

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Purpose. To investigate the possibility of delivering of anticancer drugs into the brain using colloidal carriers (nanoparticles).

Methods. Rats obtained 5 mg/kg of doxorubicin by i. v. injection in form of 4 preparations : 1. a simple solution in saline, 2. a simple solution in polysorbate 80 1% in saline, 3. bound to poly(butyl cyanoacrylate) nanoparticles, and 4. bound to poly(butyl cyanoacrylate) nanoparticles overcoated with 1% polysorbate 80 (Tween® 80). After sacrifice of the animals after 10 min, 1, 2, 4, 6, and 8 hours, the doxorubicin concentrations in plasma, liver, spleen, lungs, kidneys, heart and brain were determined after extraction by HPLC.

Results. No significant difference in the body distribution was observed between the two solution formulations. The two nanoparticle formulations very significantly decreased the heart concentrations. High brain concentrations of doxorubicin (>6 μg/g) were achieved with the nanoparticles overcoated with polysorbate 80 between 2 and 4 hours. The brain concentrations observed with the other three preparations were always below the detection limit (< 0.1 μg/g).

Conclusions. The present study demonstrates that the brain concentration of systemically administered doxorubicin can be enhanced over 60-fold by binding to biodegradable poly(butyl cyanoacrylate) nanoparticles, overcoated with the nonionic surfactant polysorbate 80. It is highly probable that coated particles reached the brain intact and released the drug after endocytosis by the brain blood vessel endothelial cells.

KEY WORDS: brain tumors; brain targeting; doxorubicin; nanoparticles; polysorbate 80.

INTRODUCTION

The blood-brain barrier (BBB), which is formed by the tight endothelial cell junctions of capillaries within the brain, limits the ability of many drugs to penetrate brain tissue and to enter the central nervous system. Malignant brain tumors respond poorly to chemotherapy, presumably because the majority of antitumor drugs cannot be delivered in effective concentrations to the tumor site. Different strategies exist for enhancement of drug delivery to the brain. Drug structural modification, usually by introduction of lipophilic groups, sometimes may enhance the brain uptake (1). Disruption of the BBB by the intracarotid infusion of a hyperosmotic solution of mannitol can significantly increase drug levels in the brain (2). Special routes of administration, such as intracarotid infusion, intervertebral arterial injection, or direct intracerebral injection are also possible; these routes, however, are associated with a high risk for the patient.

The idea of targeted drug delivery with macromolecular drug carriers is an attractive alternative, but the BBB has been for a long time considered insurmountable for large molecules and particles. A number of authors tried to use liposomes as delivery systems for the brain and reported attempts to enhance the liposomal affinity to the endothelial cells of brain blood vessels by alteration of phospholipid composition, addition of glycolipid fractions, antibody-coating (3,4,5,6), or by hyperthermic disruption of the BBB (7). The rationale for these studies was to achieve an interaction between liposomes and endothelial cells by adhesion to the cell membrane, possibly followed by fusion and subsequent release of the content into the cell. Most of these trials failed to demonstrate a remarkable efficacy.

Polymer nanoparticles have attracted considerable attention as potential drug delivery systems (8). Enhancement of therapeutic efficacy and reduction of toxicity have been demonstrated for a variety of drugs associated with nanoparticles (9). However, the predominant uptake of nanoparticles, as well as other colloidal carriers, by phagocytic cells of the reticuloendothelial system (RES) located mainly in the liver and spleen and a resulting rapid clearance from the circulation have been a major obstacle to the delivery of drugs by nanoparticles to cells, tissues, or organs other than RES (10). Several attempts have been made to change the body distribution of nanoparticles. The most promising results were obtained by coating particles with hydrophilic surfactants, which significantly altered pharmacokinetics and body distribution of nanoparticles (11) or by the covalent linkage of polyoxyethylene chains to the nanoparticles (12). Physicochemical studies have shown that coating of colloidal particles with block copolymers, such as poloxamers and poloxamines induced a steric repulsion effect, minimizing the adhesion of particles to the surface of macrophages, which in turn resulted in the decrease of phagocytic uptake and in significantly higher levels in the blood and non-RES organs, i.e. brain, intestine, kidneys, etc. (13). A number of surfactants were studied to investigate the possibility of employing poly(methyl methacrylate) nanoparticles as carriers for a delivery across the blood brain barrier (BBB) using an *in vitro* model of brain endothelial cells: Borchard *et al.* (14) showed that coating of nanoparticles with surfactants resulted in the uptake by these cells and that polysorbate 80 (Tween® 80) was the most effective surfactant for this purpose. Further *in vivo* investigations unequivocally demonstrated, that polysorbate 80-coated poly(butyl cyanoacrylate) nanoparticles loaded with the leu-enkephalin analogue dalargin enabled the delivery of this drug through the BBB and achieved a significant pharmacological effect (15,16).

In the present study, the possibility of delivery of antitumor antibiotic doxorubicin with polysorbate 80-coated nanoparticles to the brain was investigated. Doxorubicin was chosen because

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ABBREVIATIONS: BBB, blood-brain barrier; AUC, area under the time-concentration curve; RES, reticuloendothelial system.

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it is one of the most potent and versatile antitumoral agents and is known to not being able to cross the blood brain barrier under normal circumstances, i.e., i. v. injection. In addition, it binds very well to nanoparticles (17), and the doxorubicin-loaded nanoparticles were already successfully tested clinically in phase I (18).

MATERIALS AND METHODS

Materials

Doxorubicin hydrochloride, polysorbate 80 (Tween® 80) and dextran 70000 were purchased from Sigma (St. Louis, USA); iso butylcyanoacrylate was obtained from Sichel-Werke (Hanover, Germany). Other reagents used in this study were of analytical grade (Fluka Chemie, Buchs, Switzerland).

Preparation and Assay of Drug-Loaded Nanoparticles

Nanoparticles were prepared by a method of anionic polymerization according to a previously published technique (8,21). Briefly, 1% of butylcyanoacrylate was added to 1% of dextran 70 000 solution in 0.001N HCl under constant stirring. Doxorubicin was added 40 min after the beginning of polymerization to obtain a final concentration 0.4%. After 2.5 h the mixture was neutralized with 0.1 N NaOH and stirring was continued for 1 h to complete polymerization. The resulting suspension was filtered through a sintered glass filter to remove agglomerates, then spin-frozen and freeze-dried (Freeze-Drier Christ Alpha 1–5, Osterode/Harz, Germany) after addition of 3% mannitol as a cryoprotector. The particle size was measured by light scattering (Nanosizer Malvern, Great Britain); an average diameter of 270 nm was found. Doxorubicin loading to nanoparticles was calculated as the difference between total amount of drug in suspension and amount of unbound drug. For this purpose unbound drug was separated by filtration through a membrane filter (DIAFLO XM50, Amicon, Witten, Germany) and its concentration in the filtrate was measured spectrophotometrically at 495 nm. It was shown that 80% of the doxorubicin in suspension was associated with the nanoparticles. For the *in vivo* experiments the freeze-dried formulation was resuspended in saline. For surfactant coating 1% polysorbate 80 was added and the suspension was incubated for 1 h at ambient temperature under stirring prior to administration.

Animal Testing

Experiments were performed in healthy white Wistar rats 180–200 g, (Bishkek zookombinat, Bishkek, Kirgizstan). The animals had access to food and drink *ad libitum*. The Russian guidelines for the use of animals in research were followed. Animals were divided into four groups. The first and the second groups received the commercially available doxorubicin (Sigma, St. Louis, USA) diluted with normal saline, or saline containing 1% polysorbate 80 ("Dox" and "Dox+Polysorbate 80", corresp.). The third and the fourth groups were treated with the nanoparticle formulations resuspended in saline, or saline containing 1% polysorbate 80 ("Dox-NP" and "Dox-NP+Polysorbate 80"). In all groups the formulations were administered i. v. in the tail vein in a dose of 5 mg doxorubicin/kg. After selected time intervals post injection the animals (5 or 8 animals per time point as specified in the legends of the Figs. and Tabls.) were anesthesized with ethyl ether and sacrificed by decapitation. Their blood was collected, major organs removed and weighed.

HPLC Analysis of Doxorubicin

Doxorubicin levels in blood plasma and tissues were determined by HPLC. To 1 ml of blood plasma 0.5 ml of 96% ethanol was added for protein coagulation. The precipitate was separated, doxorubicin was extracted with 15 ml of a $CHCl_3$-MeOH (4:1) mixture and the solvent was evaporated. The residue was dissolved in 1 ml of MeOH and a sample (20 μl) of this solution was subjected to HPLC analysis.

Solid tissues were homogenized in the homogenizer at 90 000 rpm for 10 min. To 250 mg of the homogenized matter 4 ml of $CHCl_3$-MeOH (4:1) mixture was added and the sample was prepared as above. Doxorubicin recovery was 83% for plasma samples and 78% for tissue samples.

The extracts were subjected to the HPLC assay (HPLC apparatus Chrom-4, Laboratorni Prijistroe, Prague, Czech Republic) according to a previously described technique (19). The samples were applied to a Separon SGX C18 column (Laboratorni Prijistroe, Prague, Czech Republic), the mobile phase was acetone-phosphate buffer (pH3)-methanol (25:45:30, v/v/v), the flow rate was 0.3 ml/min. Spectrophotometric detection was performed at 495 nm. The limit of spectrophotometric detection was 50 ng/ml. When peaks on the chromatogram were too low for spectrophotometric detection the mobile phase was changed to MeOH-ammonium formate (70:30), and a fluorimetric detector (Gilson 121, Gilson Instruments, Randolph, MA) was used. Analyses were carried out at an excitation wavelength of 470 nm and an emission wavelength of 540 nm. As a result the detection limit of doxorubicin was lowered to 10 ng/ml.

The HPLC system was calibrated with intact doxorubicin. The assay was validated by determining linearity with solutions of doxorubicin hydrochloride in the appropriate concentration range. The linearity ranged from 10 ng to 25 μg and the measure of error of analysis was 2%.

Results were analyzed by the Duncan test (20) employing a significance level of $P < 0.05$.

Pharmacokinetic Analysis

The mean values for the doxorubicin concentrations and the standard errors were calculated for each time point. The AUC was calculated using a computer program "FARM" developed by Holodov L. E. and Dorohov V.V..

RESULTS

Figure 1 shows the plasma levels of the different doxorubicin formulations after i.v. administration in rats. The differences in drug levels between the nanoparticle formulations and both solutions were statistically significant except after 2 hours. Administration of doxorubicin in 1% polysorbate 80/saline solution practically does not alter the drug behaviour compared to administration in standard saline. Doxorubicin loaded to nanoparticles had a faster initial clearance from the blood. In contrast, polysorbate-coated nanoparticles led to higher plasma levels during the first two hours. This is in agreement with earlier observations by Tröster et al. (11) that showed that

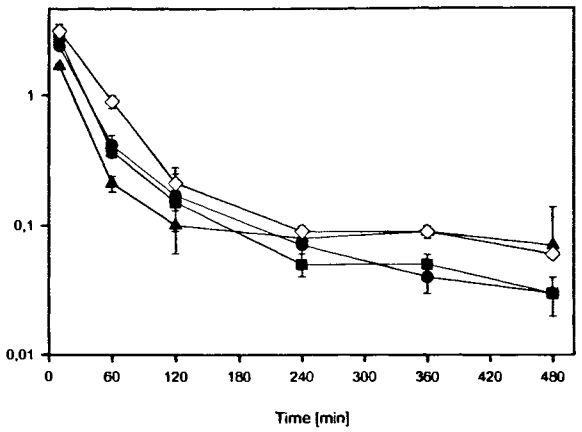

Fig. 1. Plasma levels of doxorubicin [μg/g] versus time [min]. Plasma clearance of different doxorubicin formulations in rats. Each point represents an average of 8 determinations. ● Doxorubicin (5 mg/kg) in saline, ■ Doxorubicin (5 mg/kg) in 1% polysorbate 80/saline solution ▲ Doxorubicin (5 mg/kg) bound to nanoparticles ◇ Doxorubicin (5 mg/kg) bound to nanoparticles and overcoated with 1% polysorbate 80.

coating of nanoparticles with surfactants including polysorbate 80 keeps them in the blood circulation. After this time both nanoparticle preparations were cleared from blood much slower than the solution formulations.

Pharmacokinetic parameters were calculated by fitting the data to a pharmacokinetic two-compartment open model. The values for the pharmacokinetic constants are given in Table 1. Doxorubicin pharmacokinetics was biphasic with a rapid distribution phase (α-phase) and a slow elimination phase (β-phase). A significant difference existed between free drug and the nanoparticle formulations: nanoparticle pharmacokinetics was characterized by a prominent increase in drug half-life ($T_{1/2}\beta$) and mean residence time (MRT), whereas the rate of drug transfer from the peripherical to the central compartment (k_{2-1}) was reduced. The area under the concentration-time curve (AUC) was also higher for drug bound to nanoparticles, whereas the plasma clearance (Cl) and elimination constant (K_{el}) were lower.

Although the differences in pharmacokinetic constants for drug administered in standard saline or in 1% polysorbate 80 in most cases were statistically significant, they were rather small. In contrast, this surfactant caused a substantial alteration in the behaviour of the nanoparticles: The decrease in rate constant (k_{1-2}), volume of distribution, and clearance yielded a 50% increase of the AUC value for the polysorbate-coated nanoparticles. Table 2 lists the concentrations of the doxorubicin formulations in the liver, spleen, lungs, and kidneys. Except after 10 min, generally higher concentrations were observed with the nanoparticles in organs belonging to the reticuloendothelial system (RES), i.e., liver, spleen, and lungs in comparison to free drug, which is in accordance with previously reported data (21). The accumulation in these organs was reduced, if nanoparticles were coated with polysorbate 80. (Although this reduction in some cases was statistically significant, it was not very pronounced.)

However, much more important, both types of nanoparticles prevented the accumulation of the drug in the heart (Fig. 2). Low doxorubicin levels were only found in this organ within the first hour of the experiment (0.30 ± 0.05 μg/g versus 4.27 ± 0.20 μg/g for free drug.). After that period the doxorubicin levels were below the detection limit (<0.1 μg/g). This is in agreement with earlier findings of Couvreur *et al.* (17,22) with uncoated nanoparticles.

In the brain only the polysorbate-coated nanoparticles were able to deliver doxorubicin (Fig. 3). The concentrations achieved with this formulation were very considerable, i.e., above 6 μg/g and were approximately comparable to those in the other organs investigated. No traces of drug could be found in the brain tissue of the animals in the other experimental groups. As it can be seen in Fig. 3, the highest levels of the drug in the brain were observed between 2–4 hours after administration. The major pharmacokinetic constants for brain tissue are: drug half-life $T_{1/2}\beta$ = 1.56 ± 0.77 1/h, MRT = 3.98 ± 0.94 h, AUC = 35.0 ± 3.10 h.μg/g.

Table 1. Pharmacokinetic Parameters of Dox Formulations After i.v. Administration in Rats

Parameters	Formulation			
	Dox	Dox + Polysorbate 80	Dox-NP	Dox-NP + Polysorbate 80
$T_{1/2}$ α, h	0.273 ± 0.011[a]	0.279 ± 0.020[a]	0.226 ± 0.022	0.398 ± 0.040
$T_{1/2}$ β, h	2.49 ± 0.75[a]	2.95 ± 0.60[a]	10.3 ± 1.50	8.74 ± 2.67
Mean residence time, h	1.60 ± 0.38[a]	1.93 ± 0.44[a]	9.32 ± 2.00	6.41 ± 1.84
Rate constants, 1/h:				
k_{1-2}	0.712 ± 0.033[a]	0.653 ± 0.067[a]	1.750 ± 0.180	0.602 ± 0.094
k_{2-1}	0.420 ± 0.060[a]	0.398 ± 0.066[a]	0.170 ± 0.009[b]	0.147 ± 0.052[b]
K_{el}, elimination rate constant, 1/h	1.68 ± 0.07[a]	1.76 ± 0.11[a]	1.21 ± 0.092	1.11 ± 0.15
V_c, l	1.39 ± 0.05	1.30 ± 0.06	1.65 ± 0.11	1.21 ± 0.13
Cl, clearance, ml/min	38.9 ± 1.5[a]	38.2 ± 0.8[a]	33.3 ± 2.0	22.3 ± 1.6
AUC, h.μg/ml	2.14	2.18	2.50	3.73

Note: Level of significance according to the Duncan test $p < 0.05$. This level is reached between the different groups unless indicated by superscript letters.

[a] Nonsignificant difference between DOX and DOX + polysorbate 80.

[b] Nonsignificant difference between DOX-NP and DOX-NP + polysorbate 80.

Table 2. Doxorubicin Concentrations in Liver, Spleen, Lungs, and Kidneys After i. v. Injection by Rat (n = 5)

Preparation	Drug level, μg/g					
	10 min	1h	2h	4h	6h	8h
Liver						
DOX	4.20 ± 0.64	12.40 ± 0.82	12.60 ± 0.82	5.70 ± 0.33	3.10 ± 0.40[a]	2.05 ± 0.06
DOX + PS 80	10.20 ± 1.7	14.70 ± 1.30	10.50 ± 1.20[c]	4.50 ± 0.60	2.70 ± 0.43[a]	1.71 ± 0.14
DOX − NP	6.80 ± 0.75	18.20 ± 0.90	15.80 ± 1.20	10.20 ± 0.70	6.50 ± 0.53[b]	4.80 ± 0.50
DOX − NP + PS 80	5.10 ± 0.42	19.50 ± 1.10	10.60 ± 1.30[c]	8.60 ± 0.96	6.20 ± 0.47[b]	4.00 ± 0.21
Spleen						
DOX	3.60 ± 0.50	10.50 ± 1.3	6.80 ± 0.57	4.20 ± 0.40	2.10 ± 0.13[a]	1.40 ± 0.08
DOX + PS 80	6.80 ± 0.01	11.60 ± 0.80	8.40 ± 0.22	2.80 ± 0.13	1.68 ± 0.11[a]	0.56 ± 0.10
DOX − NP	8.00 ± 0.57	20.20 ± 1.70	26.50 ± 1.45	18.90 ± 1.60	18.50 ± 1.80	10.40 ± 1.40
DOX − NP + PS 80	7.40 ± 0.61	18.00 ± 1.38	20.50 ± 1.70	16.10 ± 1.30	14.90 ± 1.50	8.20 ± 0.96
Lungs						
DOX	19.40 ± 2.20	30.10 ± 2.70[a,e]	25.30 ± 1.73	14.30 ± 1.11[a]	9.70 ± 1.30[a]	3.57 ± 0.70[a]
DOX + PS 80	12.50 ± 0.14	27.50 ± 3.2[a,e]	20.60 ± 1.20	15.30 ± 1.60[a]	10.20 ± 1.40[a]	3.40 ± 0.50[a]
DOX − NP	8.60 ± 0.25	29.50 ± 1.42[e]	42.80 ± 3.20	30.60 ± 4.40	24.20 ± 1.50	16.70 ± 1.80
DOX − NP + PS 80	5.10 ± 0.90	19.70 ± 2.10	37.20 ± 4.23	34.50 ± 3.30	20.60 ± 1.75	13.40 ± 1.40
Kidneys						
DOX	16.40 ± 1.70	40.70 ± 3.60	42.10 ± 4.27[a]	20.70 ± 2.50	16.70 ± 2.10	8.60 ± 0.62
DOX + PS 80	23.80 ± 2.46	32.90 ± 3.40[c]	40.50 ± 2.75[a]	26.80 ± 3.00[d]	20.20 ± 1.67[c]	10.10 ± 1.30
DOX − NP	8.40 ± 0.70	36.50 ± 4.30	28.30 ± 2.20	26.50 ± 1.90[d]	22.70 ± 2.40	18.50 ± 1.40
DOX − NP + PS 80	12.70 ± 1.56	32.40 ± 2.91[c]	36.50 ± 3.70	24.10 ± 1.65	20.50 ± 2.10[c]	16.70 ± 1.53

Note: DOX = Doxorubicin (5mg/kg) in saline; DOX + PS 80 = Doxorubicin (5mg/kg) in 1% polysorbate 80/saline solution; DOX − NP = Doxorubicin (5mg/kg) bound to nanoparticles; DOX − NP + PS 80 = Doxorubicin (5mg/kg) bound to nanoparticles and overcoated with 1% polysorbate 80. Level of significance according to the Duncan test $p < 0.05$. This level is reached between the different groups unless indicated by superscript letters:

[a] Nonsignificant difference between DOX and DOX + polysorbate 80.
[b] Nonsignificant difference between DOX−NP and DOX−NP + polysorbate 80.
[c] Nonsignificant difference between DOX + polysorbate 80 and DOX−NP + polysorbate 80.
[d] Nonsignificant difference between DOX + polysorbate 80 and DOX−NP.
[e] Nonsignificant difference between DOX, DOX + polysorbate 80 and DOX−NP.

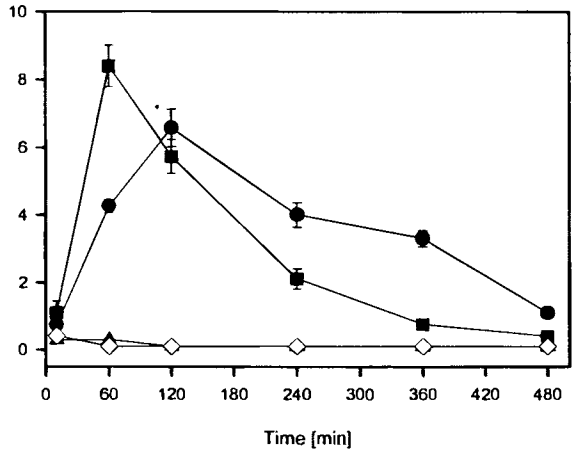

Fig. 2. Doxorubicin levels in rat heat [μg/g] versus time [min]. Uptake by rat heart of i.v. injected doxorubicin formulations. Each point represents an average of 5 determinations. ● Doxorubicin (5 mg/kg) in saline, ■ Doxorubicin (5 mg/kg) in 1% polysorbate 80/saline solution ▲ Doxorubicin (5 mg/kg) bound to nanoparticles ◇ Doxorubicin (5 mg/kg) bound to nanoparticles and overcoated with 1% polysorbate 80.

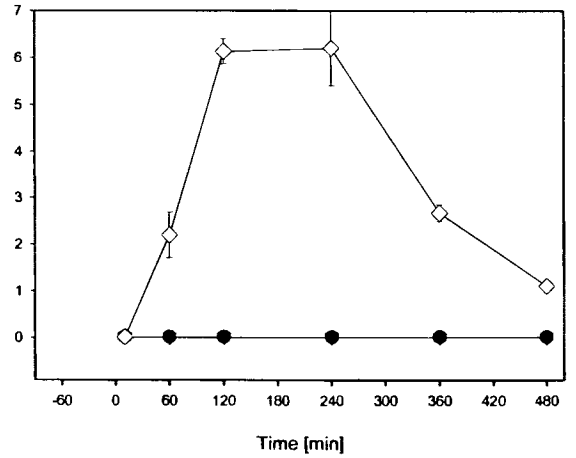

Fig. 3. Doxorubicin levels in rat heat [μg/g] versus time [min]. Uptake by rat brain of i.v. injected doxorubicin loaded to polysorbate 80-coated nanoparticles. Each point represents an average of 8 determinations. ● Doxorubicin (5 mg/kg) in saline, ■ Doxorubicin (5 mg/kg) in 1% polysorbate 80/saline solution ▲ Doxorubicin (5 mg/kg) bound to nanoparticles ◇ Doxorubicin (5 mg/kg) bound to nanoparticles and overcoated with 1% polysorbate 80.

DISCUSSION

The results of our experiments showed that polysorbate 80-coated particles were able to deliver doxorubicin to the brain of experimental animals (Fig. 3). The highest levels were achieved between 2 and 4 hours after drug administration. For the brain the mean residence time and the $T_{1/2}$ in the elimination phase were 2–3 times lower than for other tissues, which means that brain uptake was a slow but rather effective process. Administration of free doxorubicin in saline, or in 1% polysorbate 80 solution or loaded to non-coated nanoparticles could not enhance brain uptake. These data correlate with the conclusions of Kreuter *et al.* (16) that coated nanoparticles reach brain endothelial cells essentially intact so that the drug is delivered to the brain.

The possibility to transport drugs across the blood-brain barrier (BBB) with polymer nanoparticles has been demonstrated previously in a number of studies: Tröster *et al.* (11) observed that the coating of non-biodegradable poly(methyl methacrylate) ^{14}C-labeled nanoparticles with surfactants, including polysorbate 80, led to a significantly higher brain concentration of the nanoparticles after i.v. injection to rats. However, these authors at that time did not believe that the particles might have left the lumen of the brain blood vessels and were transported across the BBB. Experiments with cultivated bovine brain blood vessel endothelial cells *in vitro*, however, showed that some surfactants increased the uptake by these cells. Polysorbate 80 was the most efficient agent in this respect and was identified as a potential 'lead substance' for brain targeting (14). Later, the hexapeptide enkephalin dalargin (15,16), loperamide (23), and tubocurarine (24) were transported by the polysorbate 80-coated nanoparticles across the BBB and exhibited a strong pharmacological effect. These studies indicated that these particles were able to strongly interact with the brain blood vessel endothelial cells of mice and were taken up by these cells by endocytosis. Fluorescence and electron microscopy studies of fluorescent nanoparticles suggested that the nanoparticles reached brain tissue essentially intact. This effect seems to be limited to polysorbate 80, 60, 40, and 20, since other surfactants that were effective in the tissue cultures did not achieve a corresponding pharmacological response in animals (25). Previous (14) as well as recent unpublished additional experiments using rat brain endothelial cell line RBE 4 and primary cultures of bovine, mouse, and human brain endothelium clearly showed using radiolabeling, FITC-dextrane, or rhodamine 6 G labeled poly(butyl cyanoacrylate) nanoparticles that the nanoparticles were endocytosed after coating with polysorbate 80 but not without this coating.

The experimental technique in the present study allowed to determine the total brain concentrations. Therefore; it may be argued that the high drug concentrations in the brain homogenisate observed with the nanoparticles were due to particles remaining in the blood compartment of the brain or in the blood vessel endothelial cells of the brain. However, the tissue capillary blood volume represents only approximately 1% of the total volume of the brain and the endothelial cell volume 0.1% (26). The total concentration of the brain homogenisate in our study was 6 μg/g. If the particles containing drug had remained in the tissue blood capillaries, the resulting concentration would have been 600 μg/g, or even 6000 μg/g, assuming exclusive uptake by the endothelial cells without release into the residual brain. These concentrations are unreasonably high and would have led to immediate severe local toxicity which was not observed. In fact, the blood plasma concentration after 2 hours was only 0.21 μg/ml and after 4 hours 0.09 μg/ml. In addition, considerations with respect to doxorubicin remaining in the brain vasculature would apply equally to controls as to experimental animal group. Clearly this vascular doxorubicin did not contribute significantly to measured brain content, since levels in the control animals were below the detection limit. Therefore, we assume that due to their very similar nature to the previous nanoparticles the nanoparticles employed in the present study were taken up by a similar pathway, i.e., by endocytosis, as in the above mentioned studies. Another possibility is an opening of the tight junctions between the brain endothelial cells. However, measurements of the inulin spaces, a classical method to determine this opening (27), did not detect a major blood brain barrier opening (unpublished results). Nevertheless, besides endocytosis and opening of the tight junctions a third possibility exists for the increased brain levels of doxorubicin, namely inactivation of the P-glycoprotein efflux pump. This glycoprotein is present in the brain endothelial cells (28) and is responsible for the multidrug resistance which represents a major obstacle to cancer chemotherapy (29,30). Surfactants including polysorbate 80 were shown to inhibit this efflux system and to reverse multidrug resistance (29,30). However, as shown in this study, addition of polysorbate to the drug solution was totally inefficient. On the other hand, this surfactant, of course, may be delivered more efficiently to the brain endothelial cell if it is adsorbed to the nanoparticle surface. This could explain why polysorbate-coated nanoparticles provided high brain concentrations and not a simple surfactant solution. Nevertheless, we believe that the latter mechanism, i.e., blockage of the efflux system by polysorbate 80, may contribute to the higher doxorubicin uptake mechanism but that induction of the endocytotic uptake may play an equal or much larger role. The reason for this assumption is the observation that in contrast to other organs significant brain concentrations were obtained only after 2 to 4 hours. Such a delayed response is typical for a delivery mediated by the polysorbate-coated nanoparticles (15,16,23) and seems to be a reflection of the time-consuming process of endocytosis.

Drug-loaded nanoparticles also altered the drug distribution in other organs. Biodistribution of a drug such as doxorubicin which binds very well to the particles generally correlates with the distribution profile of the carrier. In our study nanoparticles yielded a four-fold increase in plasma half-life $T_{1/2}$ and a six-fold increase of the mean residence time (Table 1). Uptake of the drug by spleen, liver, and lungs increased by 1.5–4.8 times (Table 2), while the cardiac concentration was dramatically reduced (Fig. 2) - a phenomenon previously noted by other authors (17,18,22). On the other hand, the pharmacokinetics of the nanoparticle formulations of doxorubicin retains features that are typical for the behaviour of free drug, i.e., a biphasic decline of the plasma concentrations (Fig. 1), high rate constants for transfer from the central compartment to the peripheral, and low constants of reverse transfer (Table 1).

Coating of nanoparticles with polysorbate 80 resulted in an increase of the plasma AUC and a decrease of doxorubicin uptake by spleen, liver, and lungs by 1.5–1.7 times, as compared to the non-coated carrier, which is in a good agreement with previously obtained data.

In conclusion, our results provide evidence that the BBB is not inert but represents a highly dynamic system and that endothelial cells are capable of endocytic uptake of nanoparticles. Consequently, colloidal drug delivery systems such as nanoparticles hold promise for the transport of agents to the brain which otherwise are not capable to penetrate through the BBB. The high brain concentrations achieved in the present study may enable a significant improvement in the treatment of brain tumors. In addition, they possibly also could help to overcome multidrug resistance. If the results observed in the present study would be reproduced in humans this could open up totally new possibilities in brain cancer therapy.

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