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Availability of Liposomes as Drug Carriers to the Brain

Kazuko Kobayashi* Mei Han†

Shinobu Watarai‡ Tatsuji Yasuda**

*Okayama University,

†Okayama University,

‡Okayama Univeristy,

**Okayama University,

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Kazuko Kobayashi, Mei Han, Shinobu Watarai, and Tatsuji Yasuda

Abstract

Phospholipid vesicles, also known as liposomes, were examined for their ability to act as a drug carrier to the brain. 9-Amino-1,2,3,4-tetrahydroacridine (THA), a centrally acting acetylcholinesterase inhibitor, was used as a model drug. THA was encapsulated in dehydration-rehydration vesicles (DRV) composed of egg yolk phosphatidylcholine, cholesterol and dipalmitoyl-phosphatidic acid (molar ratio, 10/10/1) and injected into the heart of mice. The toxicity and side effects of THA were reduced by encapsulation in liposomes. The THA concentration in the mouse brain after injection of THA-encapsulated DRV at a dose of 2 mg/kg remained higher than that of free THA at the same dose. Effective concentration of THA in the brain was also prolonged by the use of liposomes, although accumulation of THA in the spleen and kidney was observed. We, therefore, concluded that liposomes are useful as carriers of drugs to the brain.

KEYWORDS: brain targeting, liposomes, mouse, THA(9-amino-1, 2, 3, 4, -tetrahydroacridin)

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Availability of Liposomes as Drug Carriers to the Brain

Kazuko KOBAYASHI*, Mei HAN, Shinobu WATARAI and Tatsuji YASUDA

Department of Cell Chemistry, Institute of Cellular and Molecular Biology, Okayama University Medical School, Okayama 700, Japan

Phospholipid vesicles, also known as liposomes, were examined for their ability to act as a drug carrier to the brain. 9-Amino-1,2,3,4-tetrahydroacridine (THA), a centrally acting acetylcholinesterase inhibitor, was used as a model drug. THA was encapsulated in dehydration-rehydration vesicles (DRV) composed of egg yolk phosphatidylcholine, cholesterol and dipalmitoylphosphatidic acid (molar ratio, 10/10/1) and injected into the heart of mice. The toxicity and side effects of THA were reduced by encapsulation in liposomes. The THA concentration in the mouse brain after injection of THA-encapsulated DRV at a dose of 2 mg/kg remained higher than that of free THA at the same dose. Effective concentration of THA in the brain was also prolonged by the use of liposomes, although accumulation of THA in the spleen and kidney was observed. We, therefore, concluded that liposomes are useful as carriers of drugs to the brain.

Key words: brain targeting, liposomes, mouse, THA (9-amino-1,2,3,4-tetrahydroacridine)

Efficient drug delivery to the brain is desirable for the therapy of senile dementia of the Alzheimer type (SDAT), malignant brain tumors and other diseases of the central nervous system (CNS). However, new drugs for the treatment of nervous system lesions are limited by their undesirable side effects (1). Moreover, the blood brain barrier (BBB) limits the ability of most drugs, as well as macromolecules and microorganisms to pass from the circulation into the brain parenchyma. Thus far, although many attempts have been made to deliver drugs to the brain, few of these attempts have met with any success (2, 3). Therefore, efficient procedures for drug delivery to the brain are actively being sought.

Liposomes are artificial vesicles composed of one or more concentric bi-layers which enclose an aqueous space. Water-soluble drugs or biomolecules are known to be accommodated in the inner aqueous space of liposomes. The potential usefulness of liposomes as drug carriers, especially in cancer chemotherapy, has attracted considerable interest during the last few decades (4). Liposomes are non-toxic, biodegradable and poorly immunogenic. In addition, the liposome-entrapped drugs are protected from enzymatic attack until they reach the target sites. The toxicity of liposome-entrapped drugs was also reported to be reduced (5, 6). Many trials have been reported on the targeting of liposomes to specific organs or cells (4, 7-9). Previously, it was shown that liposomes were incorporated into the brain across the BBB (10, 11). This property of liposomes led us to investigate whether liposomes are advantageous for the treatment of CNS diseases.

This study was performed to elucidate whether liposomes are useful carriers of drugs to the brain.

Materials and Methods

Chemicals. Egg yolk phosphatidylcholine (egg PC) (Nippon Fine Chemical, Osaka, Japan) was commercially obtained. Cholesterol (Chol), DL-α-dipalmitoylphosphatidic acid (DPPA), and 9-amino-1,2,3,4-tetrahydroacridine (THA) hydrochloride were purchased from Sigma Chemical (St. Louis, MO, USA). As a model drug, we used THA, which is a centrally acting acetylcholinesterase (AChE) inhibitor and a therapeutic agent in the treatment of SDAT (12).

Preparation of liposomes. Preparation of THA-encapsulated dehydration-rehydration vesicles (DRV) was performed according to the method of Deamer and Uster (13) with slight modifications. Egg PC (10 μmol), Chol (10 μmol), and DPPA (1 μmol) dissolved in chloroform-methanol (2/1, v/v) were dried in a conical flask under reduced pressure and stored *in vacuo*

* To whom correspondence should be addressed.

for 30 min. Then 0.5 ml of distilled water was added and the thin lipid film was dispersed by vigorous vortexing after incubation for 1 min at 50 °C. The liposomal suspension was then freeze-dried. The dried lipid mixture was hydrated with 0.2 ml of phosphate-buffered saline (PBS; pH 7.2) containing 45 mg/ml of THA. After incubation for 2 h at room temperature, the DRV suspensions were diluted with PBS (pH 7.2) and untrapped THA was removed by centrifugation at 4,000 × *g* for 10 min.

Small unilamellar vesicles (SUV) containing THA were prepared by a modification of the method developed by Mayer *et al.* (14) as follows: Lipid film composed of egg PC (30 μmol), Chol (30 μmol), and DPPA (3 μmol) was dispersed in 1 ml of 50 mM citric acid (pH 2.0). After the liposomes had been frozen and thawed for 3 cycles and sized with a 100-nm-pore filter, the pH outside of the liposomes was adjusted to 7.4 by the addition of sodium bicarbonate and the suspension was then diluted with PBS (pH 7.4). THA-loading was performed by incubation of the liposomal suspension with THA (at a final concentration of 1 mg/ml) for 1 h at 60 °C. Untrapped THA was removed by centrifugation at 100,000 × *g* for 20 min (Beckman TL-100).

For the determination of THA concentration, an aliquot (10 μl) of the liposome suspension was transferred into a glass tube, 1 ml of chloroform-methanol (1/1, v/v) was added and dried. After adding an appropriate volume of PBS (pH 7.2), fluorescence was measured using a Hitachi F-3010 fluorometric spectrophotometer (Hitachi Ltd., Tokyo, Japan) at an excitation wavelength of 325 nm and emission wavelength of 365 nm. The size of the liposomes was determined by dynamic light scattering with DLS-ARIII (Otsuka Electronics Co., Osaka, Japan).

Animal experiments and THA determination. Specific pathogen-free ICR male mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice, 6 to 7 weeks old, were anesthetized by inhalation of diethyl ether. Each group of aniamls (5 to 10 mice) received an injection of free THA or THA entrapped in liposomes, through the heart. At 30, 60, or 180 min after administration, mice were killed by the collection of blood from the heart under diethyl ether anesthesia. Then the brain, liver, spleen, and kidney were quickly taken, rinsed in PBS (pH 7.2), and weighed. The isolation and determination of THA was performed according to modified methods of Hsieh and Yang (15) and Hsu *et al.* (16). In brief, each organ was homogenized with 5 volumes of 0.5 M sodium hydroxide. The homogenate (0.6 ml) was transferred into a glass tube, and 5 ml of cyclohexane-ethyl acetate (1/1, v/v) was added. The tubes were vortexed vigorously for 45 sec, and shaken for 10 min on a rotary shaker at room temperature. After centrifugation at 1,200 × *g* for 10 min, the organic phase was collected and dried. Then it was reconstituted with 1 ml of a mobile phase, which was prepared by mixing 410 ml of 0.1 M phosphoric acid solution (adjusted to pH 2.8 by triethylamine) and 90 ml of acetonitrile. The amount of THA was determined by high-performance liquid chromatography (Shimadzu LC-7A, Shimadzu Co., Kyoto, Japan), employing a TSK gel ODS-80Ts column (150 mm × 4.6 mm I.D.; Tosoh Co., Tokyo, Japan). Serum samples (0.3 ml each) were mixed with 0.5 ml of 0.5 M sodium hydroxide and prepared by the same procedure as above.

Statistical analysis. Results are expressed as the arithmetic mean with the standard error of the mean (mean ± SEM). Statistical differences between pairs of groups were calculated by Welch's *t*-test and those among three groups or more by the Bonferroni multiple test. Categorical data were analyzed by a contingency table method (chi-square test and Fisher's exact probability test). Less than 0.05 of *P* value was taken as the level of significance.

Results and Discussion

Survival rate of mice injected with THA. We investigated the survival rate of mice injected with free (unencapsulated) THA or THA encapsulated in liposomes. As shown in Table 1, injection with free THA resulted in 88 % and 59 % survival at the doses of 0.5 and 2 mg/kg, respectively. On the other hand, injection with THA-loaded DRV resulted in 88 % survival at the dose of 2 mg/kg. The differences between the survival rates of mice injected with free THA 0.5 mg/kg and that with free THA 2 mg/kg, and between free THA 2 mg/kg and THA-loaded DRV 2 mg/kg were statistically significant. The surviving mice at the dose of 2 mg/kg of free THA showed decreased locomotion (52 %) and tetany (17 %), whereas the surviving mice after injection with THA encapsulated in DRV (2 mg/kg) did not show such symptoms. These results clearly indicate that the toxicity and side effects of THA were reduced by encapsulation in liposomes. There are many reports that liposomal encapsulation reduces the toxicity of the drug

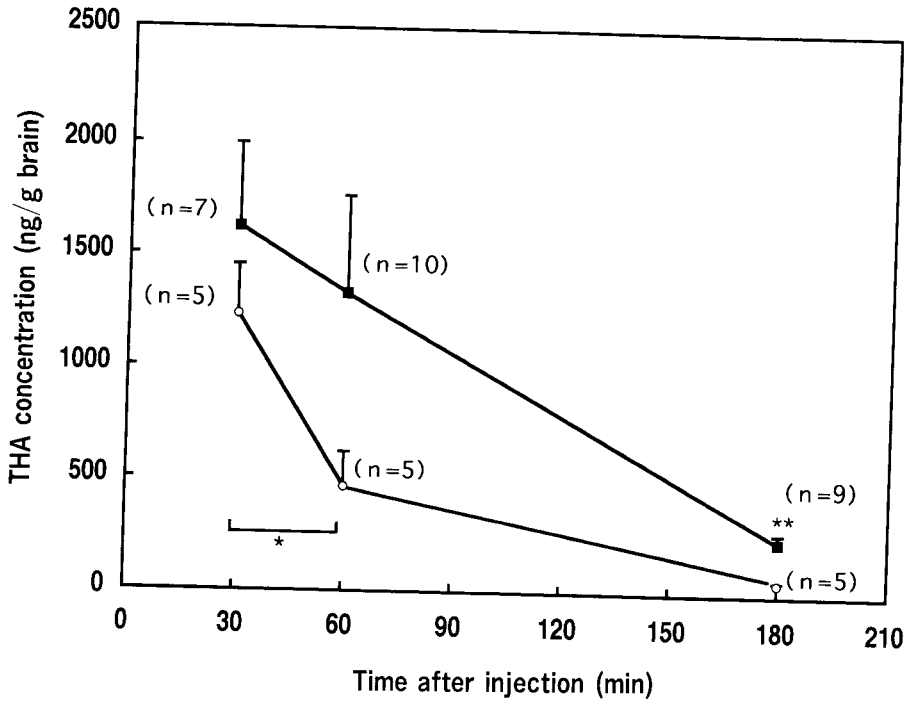

Fig. 1 9-Amino-1,2,3,4-tetrahydroacridine (THA) concentration in mouse brain at 30, 60, and 180 min post-injection of free THA and THA encapsulated in dehydration-rehydration vesicles (DRV). Free THA (2 mg/kg: ○) or THA encapsulated in DRV (2 mg/kg: ■) were injected into the heart of mice. Mice were killed by blood-collection from the heart 30, 60, and 180 min after injection and the brains were collected. The THA concentration in the brain was determined as described in "Materials and Methods". Results are expressed as mean ± SEM. The number of mice tested are indicated in the parentheses.
* $P < 0.05$, between the concentration of THA in mouse brain at 30 min and at 60 min post-injection of free THA (Bonferroni test).
** $P < 0.01$, between the concentration of THA in mouse brain at 180 min post-injection of free THA and that of THA encapsulated in DRV (Welch's t-test).

Table I Survival rate in mice injected with THA

Drug form	Dose (mg/mg)	Survival rate (%)	
Free THA	0.5	15/17 (88)	*
Free THA	2	23/39 (59)	*
THA in DRV	2	21/24 (88)	

THA: 9-Amino-1,2,3,4-tetrahydroacridine; DRV: Dehydration-rehydration vesicles.
* $P < 0.05$.

(5, 6). Our results agree with these previous reports.

THA concentration in the mouse brain. In order to explore whether free THA and liposomal THA were incorporated into brain tissue when injected into the heart of mice, we analyzed the THA concentration in the mouse brain at 30 min after administration. The THA concentration in the brain of mice injected with THA encapsulated in DRV at the dose of 2 mg/kg was 1623 ± 373 ng/g wet weight of brain (n=7), whereas that of mice injected with free THA (0.5 mg/kg) was 334 ± 70 ng/g brain (n=6). The difference between these two types of administration was statistically significant ($P < 0.05$), while the survival rates of these two groups of mice were comparable. The time-course of the THA concentration in the mouse brain after the injection of free THA and THA encapsulated in DRV at the dose of 2 mg/kg was also studied, and the results are shown in Fig. 1. At 30 min after the injection of free THA, the concentration of THA was 1233 ± 221 ng/g wet weight of brain and it was significantly lower at 60 min. On the other hand, after the injection of THA entrapped in DRV, the brain concentration of THA was highest at 30 min (1623 ± 373 ng/g), and the difference between the brain concentration at 30 min and that at 60 min was insignificant. At 180 min post-injection, the

Table 2 Organ distribution of THA in mice 60 min after administration of free THA and THA in the liposomal formulation

Drug form	Number of mice tested	THA concentration (ng/g wet weight)				
		Brain	Liver	Spleen	Kidney	Serum
Free THA	5	467 ± 154[a]	256 ± 36	577 ± 263	2385 ± 784	31 ± 6
THA in DRV	10	1328 ± 436	423 ± 116	1529 ± 292	2110 ± 706	105 ± 43
THA in SUV	8	273 ± 11	244 ± 41	2418 ± 136	1193 ± 152	48 ± 12

Mice were injected with free THA, THA encapsulated in DRV (mean size; about 3 μm), and THA encapsulated in SUV (mean size; about 150 nm) at the dose of 2 mg/kg. The mice were sacrificed 60 min after injection and each organ was collected. THA concentration and liposomal size were determined as described in "Materials and Methods".
SUV: Small unilamellar vesicles; THA, DRV: See Table 1.
[a]: Mean ± SEM.

THA concentration in the brain of mice injected with THA encapsulated in DRV was significantly higher than that of mice injected with free THA ($P < 0.01$). This may be due to the sustained-release effects of liposomes (17).

Nielsen *et al.* reported that THA was effective in reversing amnesia induced by scopolamine assessed in T-maze tests in rats and passive avoidance tests in mice following an intraperitoneal injection of THA 3.2 mg/kg (18). The concentration of THA in the brain of mice and rats needed to be above 230 ng/g wet weight of brain to achieve AChE inhibition (18). In this study, the THA concentration in the mouse brain after injection of THA-containing liposomes remained higher than that of free THA. The concentration of THA in the brain following an injection of free THA (2 mg/kg) remained above 230 ng/g wet weight of brain for about 120 min, while that following an injection of THA encapsulated in DRV (2 mg/kg) was more than 180 min. Furthermore, it should be noted that the survival rate at the dose of 2 mg/kg of free THA was 59 %, whereas that of THA at the same dose encapsulated in liposomes was 88 % (Table 1, $P < 0.05$). These results suggest that the delivery of THA to the brain can be enhanced by the use of liposomes, and that entrapment of THA into the liposomes reduced the toxic effect and prolonged the effective concentration of THA (230 ng/g wet weight of brain).

THA distribution in mouse organs. We studied the distribution of THA in mouse organs 60 min after the administration of free THA and DRV-entrapped THA (Table 2). When free THA (2 mg/kg) was injected into mice, the THA content in organs such as the brain, liver, and spleen was low but THA accumulation was marked in the kidney. On the other hand, in mice injected with THA entrapped in DRV (2 mg/kg), THA accumulated not only in the brain, but also in the spleen and kidney. THA also accumulated in the liver but to a lesser extent. Moreover, the important observation that liposomal size strongly influences relative tissue uptake has recently been followed up (19). Therefore, the effect of reducing the liposomal size on the THA distribution in mouse organs was also examined. The result, given in Table 2, shows that mainly the administered THA-entrapped SUV liposomes accumulate in the spleen and kidney. After an administration of SUV, we did not find a remarkable accumulation of THA in the brain. In the serum, only a small amount of THA was retained irrespective of the administration of THA in the free form or in the liposome-entrapped form.

Schackert *et al.* have reported that the injection of liposomes into the internal carotid artery of mice could allow accumulation of drug in the brain (20). The highest accumulation of liposomes in the brain occurred with large multilamellar vesicles ($> 5\,\mu$m). Smaller liposomes ($< 1\,\mu$m, 40–80 nm) were not toxic but did not accumulate in the brain, either. In the present study, after an injection of THA encapsulated in DRV, whose mean size was about 3 μm, the THA concentration in the brain homogenate was higher than with free THA. On the other hand, it was not increased after an injection of THA encapsulated in SUV, whose mean size was about 150 nm. These results coincide with the report of Schackert *et al.*, and it is suggested that the smaller liposomes do not accumulate in the brain. In general, most of liposomes intravenously injected into animals are rapidly taken up by the phagocytic cells of the reticuloendothelial

system (RES) (21). In the present study, THA-containing liposomes are accumulated in the spleen and kidney (Table 2). More recently, it has been reported that RES-avoiding liposomes had a longer serum half life in normal mice (22, 23) and accumulated in tumors in tumor-bearing mice (24, 25). Thus, if THA-containing liposomes are able to avoid RES uptake, it would be expected that they are taken up significantly by the brain. We are now investigating the usefulness of RES-avoiding liposomes as a vehicle for the delivery of drugs to the brain. Furthermore, it is of interest that although THA concentration in the serum was very low in all three formulations, *i. e.*, free THA, THA encapsulated in DRV, and THA encapsulated in SUV, the concentration of THA in the brain following the injection of THA encapsulated in DRV was much higher than the others. Although it seems likely that this is due to the penetration of the drug into the parenchyma of the brain, this has not yet been confirmed experimentally. Studies are currently underway on the penetration of THA into the parenchyma of the brain.

The potential usefulness of liposomes as drug carriers has recently attracted considerable interest (4, 26). The present study clearly indicated that the toxicity and side effects of THA were reduced by encapsulation into liposomes and also that the THA concentration in the brain following an injection of liposome-entrapped THA was much higher than that following an injection of free THA. In addition, the effective concentration of THA in the brain was also prolonged. These results strongly suggest that liposomes are useful as carriers of drugs to the brain.

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