African Journal of Pharmacy and Pharmacology Vol. 3(5). pp. 227-233, May, 2009
Available online http://www.academicjournals.org/ajpp
ISSN 1996-0816 © 2009 Academic Journals

Full Length Research Paper

PAMAM-drug complex for delivering anticancer drug across blood-brain barrier *in-vitro* and *in-vivo*

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Accepted 22 April, 2009

The delivery of anticancer drugs to the brain is profoundly limited by blood-brain barrier (BBB). The purpose of this work is to develop a new carrier for doxorubicin to overcome the BBB. Polyamidoamine (PAMAM) dendrimer, a novel nanoscopic high-branching polymer, was exploited as an efficient carrier of doxorubicin. The MTT assay showed that PAMAM (generation 3) had little cytotoxicity in brain capillary endothelial cells (BCECs). The results of fluorescence intensity assay and fluorescent microscopy showed that the cellular uptake of PAMAM/doxorubicin complex was much higher than that of free doxorubicin and exhibited concentration and time dependent manners. The action of PAMAM in increasing the cellular uptake of doxorubicin was stronger than that of verapamil, a P-glycoprotein (P-gp) inhibitor. In body distribution study, the brain uptake of doxorubicin in PAMAM/doxorubicin group increased dramatically (about 6-fold) compared to that in free doxorubicin group. These data suggest that the novel PAMAM/drug complex is a simple but efficient system, which showed great capability to cross the BBB. PAMAM dendrimer could be used as an effective carrier to deliver anticancer drugs to the brain.

Key words: PAMAM, doxorubicin, blood-brain barrier, p-glycoprotein.

INTRODUCTION

A major challenge in the management of diseases in central nervous system (CNS) is the limited penetration of therapeutic drugs into the brain. The barrier responsible is the capillaries of the brain, whose endothelial cells form the so-called blood-brain barrier (BBB). The brain capillary endothelial cells (BCECs) are characterized by having tight continuous circumferential junctions, which restrict the entry of most polar solutes to the brain from periphery. In addition, the permeation of compounds is considered to be limited by the extruding action of active efflux transport proteins including P-glycoprotein (P-gp) and multidrug resistance proteins (MRPs) which are highly expressed in BCECs (Pardridge, 2003). Besides the development of simple prodrugs, an emerging approach to circumvent the BBB is the development of liposomes, polymeric complexes, nanoparticles or solid lipid nanoparticles, in which the therapeutic drugs can be adsorbed or entrapped (Jain et al., 2007; Kuo et al., 2008). Polymeric drug carriers of nano-scaled size range have attracted increasing attention in recent years. The use of polymeric complexes may effectively disguise the membrane barrier to drug molecules (Costantino et al., 2005).

PAMAM dendrimer, a class of novel nano-scaled polymers, is considered an appropriate option. PAMAM is spherical and has well-defined tree-like structures with extraordinary symmetry, high branching and maximized terminal functionality density (Caminade et al., 2005). Furthermore, PAMAM has demonstrated its potential use as a drug delivery system. A variety of molecules, such as drugs and other therapeutic agents, can be embedded in the interior void space of PAMAM or adsorbed on the cationic surface to increase their cellular uptake (Ke et al., 2008; Fant et al., 2008). PAMAM is capable of complexing with DNA and oligonucleotides and have been proven to enhance the cytosolic and nuclear uptake of nucleic acids (DeMatti et al., 2004; Dufès et al., 2005; Huang et al., 2008; Nam et al., 2009). Moreover, PAMAM has been reported as a solubility enhancer of drugs such as ketoprofen (Yang et al., 2004; Cheng et al., 2005) and shown the potential in controlled drug delivery Papagiannaros et al., 2005). (In addition, increasing evidence showed an important property of PAMAM, transepithelial

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and endothelial transport ability, which is of great interest to pharmaceutical researchers (Jevprasesphant et al., 20 03; Kitchens et al., 2005).

At present, doxorubicin is widely selected as a model anti-tumor drug. Doxorubicin is an anthracycline antibiotic that is of increasing importance in the treatment of brain malignant tumors (Lesniak et al., 2005). Still, due to the presence of the BBB, systemic doxorubicin does not penetrate into the brain to reach effective concentrations for producing anti-tumor action. Furthermore, intravenous administration (i.v.) of high-dose doxorubicin is often associated with acute toxicity, such as myelosuppression, immunosuppression and dose-cumulative cardiotoxicity. The distribution of doxorubicin into the brain is also restricted by P-gp on the BBB under normal physiological conditions when doxorubicin is considered to be a typical substrate to P-gp (Tilloy et al., 2006; Abu et al., 2009).

To circumvent the brain limited access of doxorubicin, differrent approaches were developed such as liposome or nanoparticle drug delivery systems and peptide-vector strategy using doxorubicin linked to cationic peptides (Chen et al., 2004; Schally, 2008). More invasive method such as temporary disruption of the BBB via osmotic pressure modification could increase the accumulation of doxorubicin in the brain (Kondo et al., 1987). However, a proper approach is highly desired for delivering doxorubicin into the brain tumors through non-invasive intravenous administration.

To overcome the BBB, we tested the capability of PAM AM that is able to cross cellular membranes to deliver doxorubicin in P-gp-expressing BCECs. The characteristics of PAMAM/doxorubicin *in vitro* and the biodistribution in balb/c mice were evaluated.

EXPERIMENTAL

Materials

Polyamidoamine (PAMAM) dendrimer of generation 3, Triton X-100, TES [N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid] and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin hydrochloride was a generous gift from Haizheng medical drug Co. (Zhejiang, China). Verapamil was obtained from Hengrui company (Jiangsu, China). Other reagents, if not specified, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethics committee of Fudan university.

Cell culture

Primary BCECs were isolated from cerebral gray matter of balb/c mice and routinely cultured as described previously (Huang et al., 2007). Briefly, BCECs were cultured at 37°C under a humidified atmosphere containing 5% CO_2 in special Dulbecco's modified eagle's medium ($DMEM_F$) supplemented with 20% heat-inactivated fetal calf serum (Gibco), 100 μg/ml epidermal cell growth factor, 2 mM L-glutamine 20 μg/ml heparin, 40 μU/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. All the cells used in this study were between passage 10 and passage 15.

Preparation of PAMAM/doxorubicin complex

The PAMAM/doxorubicin complex was prepared as described previously (Papagiannaros et al., 2005). Briefly, a given amount of doxorubicin and PAMAM (2:1, mol/mol) were mixed. The solution was dissolved in TES solution (10 mM, pH 7.5) and stirred overnight at room temperature. Complexes used in the following experiments were freshly prepared.

Characterization of PAMAM/doxorubicin complex

The mean diameter and zeta potential of the complex was measured by dynamic light scattering using a Zeta potential/particle Sizer NicompTM 380 ZLS (PSS Nicomp particle size system, U.S.A.).

The MTT cytotoxicity assay of PAMAM

For cytotoxicity assay, BCECs were seeded at a density of 4.5×10^3 cells/well in 96-well plates (corning-coaster, Japan) for 96 h. PA MAM was dissolved in phosphate buffer solution (PBS, pH 7.4) to a series of solutions and then added to wells. Final concentrations of PAMAM in the wells were adjusted and ranged from 0.1 to 50 μM. After 2 h incubation, the media were discarded and 100 μl of the MTT solution (0.5 mg/ml in PBS) was added to each well. The incubation with MTT solution was performed at 37°C for 2 h, at the end of which the media were discarded. Then 100 μl of DMSO solution was added into each well to solubilize formazan crystals. The culture plates were placed on an orbital shaker at 37°C for 10 min. The absorbance was measured at the test wavelength of 570 nm and the reference wavelength of 630 nm by a microplate reader (Bio-Rad, TOKYO, Japan). Cells without the addition of PAMAM solution served as positive controls. Assays with each PAMAM concentration were repeated 4 times.

Fluorescence intensity assay

BCECs were seeded at a density of 2×10^4 cells/well in 24-well plates (Corning-Coaster, Japan), incubated for 72 h and checked under the microscope for confluency and morphology. Rinsed twice with TES solution, BCECs were incubated with PAMAM/doxorubicin complex or free doxorubicin solution in a doxorubicin concentration range of 0.5 to 50 μM. After incubation at 37°C for 60 min, BCECs were washed 3 times with TES solution and further incubated with Triton X-100 (1%, w/v) overnight for cellular lysis. The fluorescence intensity of doxorubicin (excitation at 537 nm and emission at 584 nm) was assessed by a microplate fluorometer (Perkin-Elmer) and the cellular uptake was calculated through a fluorescence intensity-concentration standard curve. Protein concentrations of the cell lysates were determined by the Bradford colorimetric assay with bovine serum albumin (BSA, Pierce) as a protein standard (Bradford, 1976). The cellular uptake of doxorubicin was then normalized to protein amounts to ng/μg cell protein. For time course study, BCECs were incubated with 3 kinds of drug solutions, free doxorubicin solution, doxorubicin solution with the addition of 100 mg/ml Verapamil, PAMAM/doxorubicin complex solution, respecttively. The doxorubicin concentration was fixed in 10 μM. Incubation was performed at 37°C for up to 90 min. The cellular uptake of doxorubicin was calculated as described above each concentration was performed in quadruplicate.

Fluorescent microscopy

BCECs were cultured and incubated with 3 drug solutions descrybed above. The concentration of doxorubicin was fixed in 10 μM

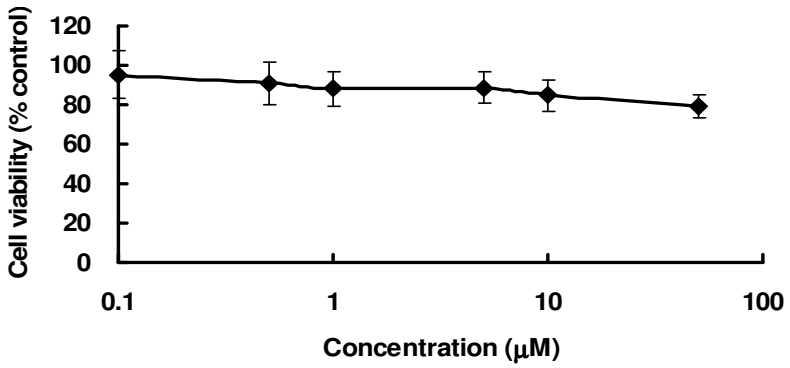

Figure 1. The cytotoxic effects of PAMAM at concentrations 0.1~ 50 μM on BCECs following 2 h exposure as determined by the MTT assay. Data represent the mean ± standard error of mean (S.E.M.) (n = 4).

and 50 μM, respectively. After 60 min incubation, the fluorescence of doxorubicin was visualized under a fluorescent microscope (Olympus, Japan).

Tissue distribution study

In the biodistribution study, 0.3 ml of TES solution containing free doxorubicin solution or PAMAM/doxorubicin complex solution at a dose of 6 μg doxorubicin/g body weight were injected via the tail vein of balb/c mice. At 60 min after injection, the mice were decapitated and the organs including brain, heart, liver, spleen, lung and kidney were extirpated. The organs were carefully washed with distilled water, weighed and then homogenized. The fluorescence intensity of doxorubicin inhomogenized tissue was measured and the concentration was calculated through a fluorescence intensity-concentration standard curve as described above. Statistical analysis was performed by t-test using Stata (version 7.0) software. Animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethical committee of Fudan Uni-versity.

RESULTS AND DISCUSSION

The discovery that PAMAM dendrimer can be used successfully to deliver biologically active substances into live cells has provided the basis for developing new effective strategies for drug delivery across cellular membranes. Herein, PAMAM was used to complex with doxorubicin and then enhance the uptake of doxorubicin in BCECs *in vitro* and in mouse brain *in vivo*. Using the intrinsic fluorescence of doxorubicin, the cellular uptake behavior and body distribution of PAMAM/doxorubicin complex were investigated compared to free doxorubicin.

Characterization of PAMAM/doxorubicin complex

The mean diameter of the complex was 3.3 ± 0.2 nm, while the zeta potential value was 7.68 ± 0.32 mV. The diameter of the complex is around 3 nm, which is similar to PAMAM (generation 3) itself (Sharma et al., 2005), demonstrating that doxorubicin was embedded in the interior void space of PAMAM. As known, amphipathic property is an important feature of PAMAM, which means that PAMAM could be exploited as an efficient carrier for small molecular hydrophobic drugs such as doxorubicin and paclitaxel. Furthermore, the complex exhibited positively charged finally, which showed its ability to bind to negative cellular membranes and should enhance the cellular uptake of doxorubicin.

MTT cytotoxicity assay

The cytotoxic activity of PAMAM was tested *in vitro* against BCECs by the MTT assay. As shown in Figure 1, the concentration of PAMAM ranged from 0.1 to 50 μM didn't cause strong cytotoxicity. When the concentration of PAMAM was 5 μM (indicating 10 μM of doxorubicin incorporated in PAMAM/doxorubicin complex), the inhibition of cellular proliferation was 11.3%. The cytotoxicity of cationic PAMAM is thought to be the result from the interaction between positively charged dendrimer and negatively charged cell surface (Malik et al., 2000; Jevprasesphant et al., 2004). In addition, several lines of evidence indica-te that higher generation of PAMAM leads to higher toxicity to cells (Malik et al., 2000). The concentration of PA-MAM was no more than 25 μM in the following experiments.

Cellular uptake experiments

At pH 7.5 (TES buffer), the incorporation efficiency of doxorubicin into PAMAM was more than 90%. Within 60 min, the release of doxorubicin from PAMAM/doxorubicin complex was quite slow and less than 10% (data not shown). The results of cellular uptake experiments showed that the uptake of free doxorubicin maintained at a low level when the concentration of doxorubicin increased from 0.5 to 50 μM after 60 min exposure (Figure 2). This might attribute to that doxorubicin is a substrate of P-gp expressed on BCECs (Zhang et al., 2003). P-gp is a

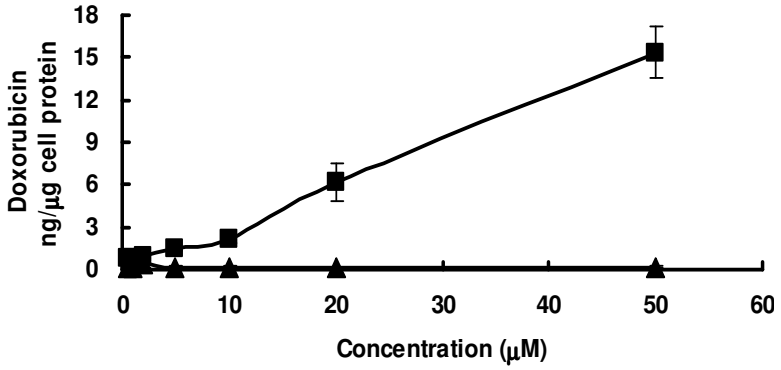

Figure 2. Cellular uptake of free doxorubicin (▲) and PAMAM/doxorubicin (■) in BCECs after 60 min incubation at 37°C. Data represent the mean ± S.E.M. (n = 4).

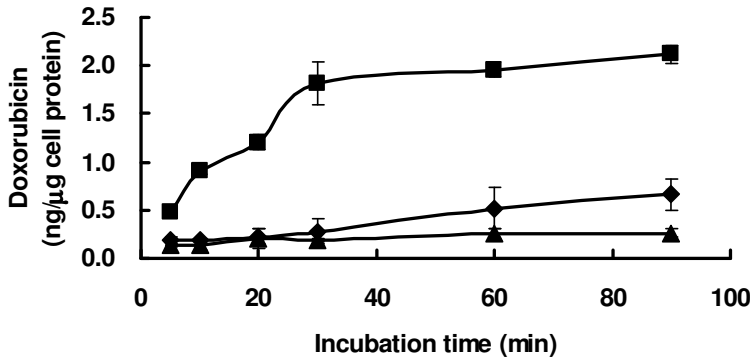

Figure 3. Time course study of free doxorubicin (▲), doxorubicin plus verapamil (∅) and PAMAM/doxorubicin (■), in a fixed concentration of doxorubicin at 10 μM, up to 90 min at 37°C. Data represent the mean ± S.E.M. (n = 4).

transmembrane protein that acts as an ATP-driven efflux pump for a wide variety of chemotherapeutic drugs from cytoplasm and thereby reducing the intracellular drug content to a sublethal level (Ambudkar et al., 2003; Barancík et al., 2006). The presence of P-gp on BCECs prevents the intracellular accumulation of free doxorubicin. However, after the same exposure period, BCECs treated with PAMAM/doxorubicin complex exhibited fluorescence intensity increasing with the concentration of doxorubicin, indicating that complexation of doxorubicin inside PAMAM could avoid the drug efflux by P-gp (Figure 2).

Many experiments have attempted to block the efflux activity of P-gp with P-gp inhibitors (Constantinides et al., 2007; Dong et al., 2008). Verapamil is a well-known specific inhibitor of P-gp (La et al., 2003). The results from the time course study showed that the cellular content of free doxorubicin maintained at a low level up to 90 min (Figure 3). The intracellular doxorubicin increased with the addition of verapamil. In addition, the intracellular amount of doxorubicin further increased markedly when incorporated in PAMAM. The similar results were observed using the fluorescent microscope (Figure 4). The intracellular fluorescence intensity of PAMAM/doxorubicin complex was much stronger than that of free doxorubicin with or without the addition of verapamil. When the concentration of doxorubicin in the complex increased to 50 μM, the fluorescence was clustered and the morphology of BCECs changed, indicating the high intracellular content and strong cytotoxicity of PAMAM/doxorubicin (Figure 4). The much higher cytotoxic activity of PAMAM/doxorubicin complex over free doxorubicin implies that the cellular uptake of doxorubicin from PAMAM/doxorubicin complex includes different transport mechanism other than simple diffusion. This result was in consistent with the cytotoxicity assay. The possible explanations of the highest accumulation of PAMAM/doxorubicin complex in BCECs are (1) the cationic PAMAM can bind with the negative cellular surface to enhance the uptake of doxorubicin and (2) doxorubicin complexed with PAMAM can avoid the recognition of P-gp and thus escape the efflux

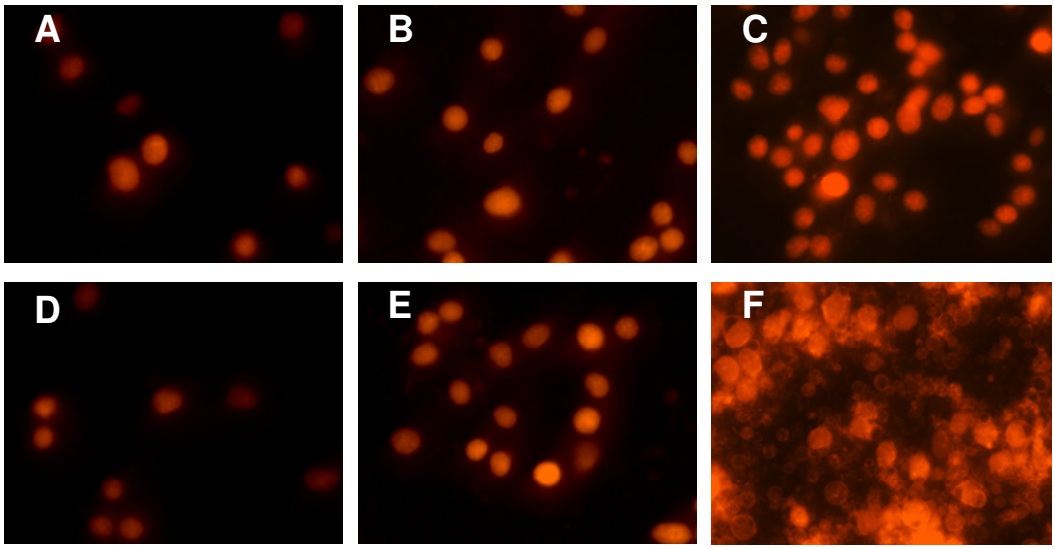

Figure 4. The fluorescent images of free doxorubicin (A, D), doxorubicin plus verapamil (B, E) and PAMAM/doxorubicin (C, F), with fixed concentrations of doxorubicin at 10 μM (panels A - C) and 50 μM (panels D-F), respectively. The incubation was performed at 37°C for 60 min. Red: intrinsic fluorescence of doxorubicin. Original magnification: 200×.

by P-gp.

The tissue distribution study

The results of tissue distribution study showed that the brain uptake of doxorubicin in the PAMAM/doxorubicin group increased dramatically (about 6-fold) compared with that in the free doxorubicin group. The likely mechanisms for the increased brain doxorubicin levels consist of 2 aspects. On 1 hand, the route PAMAM/doxorubicin enters the cells may be changed from simple diffusion to the adsorptive-mediated endocytosis, for the cationic PA MAM can bind with the anionic cellular membranes through electrostatic interaction. On the other hand, PAMAM /doxorubicin may help doxorubicin avoid the recognition and efflux of P-gp. The hypothesis is supported by a recent study which suggests that PAMAM is not a P-gp substrate and is therefore not subjected to the P-gp efflux system (El-Sayed et al., 2003). The definite mechanism(s) of endocytosis, subcellular trafficking and localization, as well as interaction with efflux pumps need further investigation. In addition, the doxorubicin level in the brain after i.v. administration of free doxorubicin was much lower than that in other organs. It is generally thought that the BBB, including the tight junction between the adjacent endothelial cells and the excessive expression of P-gp, severely interrupts the movement of small molecules (Huber et al., 2001; Wolburg et al., 2002).

After i.v. administration of PAMAM/doxorubicin complexes, the doxorubicin concentration in the liver is also significantly increased ($p < 0.001$) compared to that i.v. administration of free doxorubicin, whereas the concentrations in the spleen and kidney were not changed markedly ($p > 0.05$) (Figure 5). An interesting finding was that the concentration of doxorubicin in the heart was significantly decreased ($p < 0.001$) when doxorubicin was complexed with PAMAM. This would be beneficial in reducing cardiotoxicity which is considered as the major side effect of doxorubicin (Ferreira et al., 2008. Using free doxorubicin as contrast, PAMAM/doxorubicin complex increased the liver doxorubicin level by 1.9-fold and decreased the heart doxorubicin level by 50% at 1 h after injection.

From the results of this study, encapsulating doxorubicin into PAMAM may well modify its biodistribution pattern. It is consistent with the report that after administration into the body, nanoparticles containing doxorubicin were taken up selectively by the reticuloendothelial system (RES), particularly the liver and spleen and significantly decreased the doxorubicin concentrations in the heart (Chen et al., 2004). The active uptake mechanisms such as phagocytosis by Kupper cells can be attributed to the increased accumulation of PAMAM/doxorubicin in the liver. However, why the con-centration of PAMAM/doxorubicin in the spleen (still 1 of the major RES uptake organs) was decreased is still un-der investigation.

Conclusions

The high-branching PAMAM was investigated as an anticancer drug carrier. The cellular uptake of PAMAM/doxorubicin was in doxorubicin concentration and time dependent manners against BCECs. Compared to free doxorubicin, the distribution of PAMAM/doxorubicin in the mouse brain was significantly increased while that in the heart was decreased. The results of this study indicated that PAMAM appears to be a promising drug transport carrier to the brain.

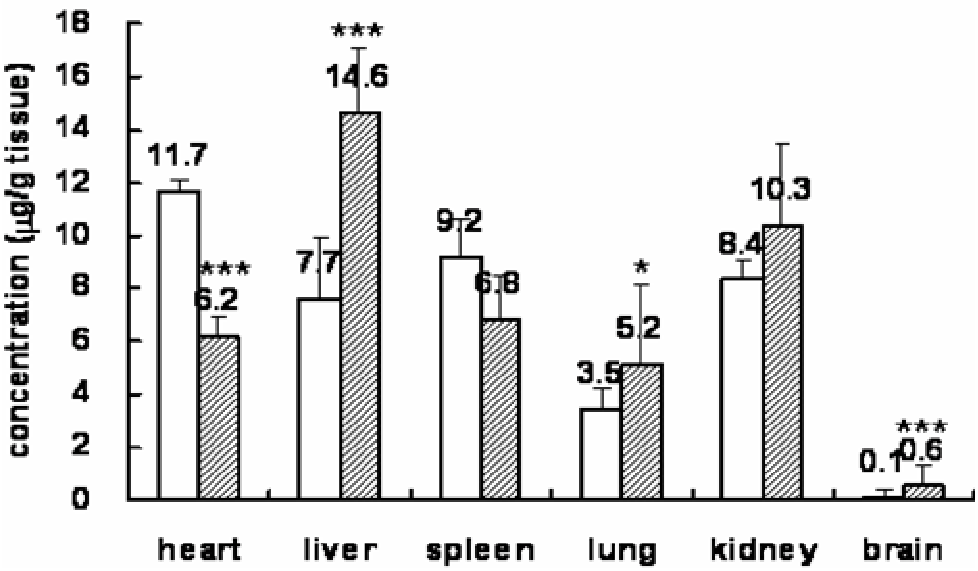

Figure 5. Tissue distribution of free doxorubicin (open column) and PAMAM/doxorubicin (filled column) in balb/c mice at a dose of 6 mg/kg. The significance of each result was compared with free doxorubicin using studeznt's-t test. *: $P < 0.05$; ***: $P < 0.001$. Data represent the mean ± S.E.M. (n = 4).

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