Proceedings of the 28th IEEE
EMBS Annual International Conference
New York City, USA, Aug 30-Sept 3, 2006

Enhanced efficacy of anti-tumor liposomal doxorubicin by hyperthermia

Ping Liu[1], Lisa Xuemin Xu[1, 3] , Aili Zhang[1, *]

Abstract—**The efficiency of novel tumor chemotherapeutics could be increased using targeted drug delivery by hyperthermia. In this paper, the 3D liposomal doxorubicin distribution in the tumor tissue enhanced by local hyperthermia was quantitatively studied in real time using laser confocal microscopy. Results showed that the thermally induced liposomal doxorubicin extravasation was non-uniform and more excessive in the peripheral region than that in the tumor center. The effect of the thermally targeted drug delivery was also investigated. On the 1st, 3rd day after the thermally targeted drug treatment, histological examination showed that many nucleolus were condensed and collapsed in the peripheral region. But, in the tumor center, there were no such changes found until the 3rd day. While on the 6th day, tumor cells in both the peripheral and center region were found necrotic. The enhancement of the nanoparticle anti-tumor drug effect was significant. A theoretical analysis of liposomal doxorubicin diffusion to the tumor cells in vivo was performed. Results showed that it took more than 40hrs for the doxorubicin to get into the tumor cells in the center region from the periphery region. The theoretical results well explained the experimental observations.**

INTRODUCTION

IN chemotherapy, many anti-tumor drugs following oral or intravenous administration have to cross many normal organs and tissues to reach the tumor site [1], resulting in large volume of anti-tumor drugs distributed in most of the tissues of the body [2]. Because of their strong toxicity, anti-tumor drugs can bring undesirable side effects on the healthy organs and tissues.

Liposome has been proposed to be useful drug carrier for targeted drug delivery system and is now considered to be a mainstream drug delivery technology that improves drug pharmacokinetics, and increases drug accumulation in tumor [3,4]. PEG (poly-ethylene glycol)-coating of liposome inhibits RES-mediated clearance, and has a prolonged circulation time [5-8] and reduces drug toxic side effects. Drugs encapsulated into PEG-liposome increase therapeutic efficacy compared to free-drugs or drugs encapsulated in the conventional liposome [9].

Ping Liu and Aili Zhang are with School of Life Science and Technology, Shanghai Jiao Tong University, Shanghai, P. R. China.

Lisa X. Xu was with School of Mechanical Engineering and Dept. of Biomedical Engineering, Purdue University, West Lafayette, IN, USA. She is now with School of Life Science and Technology, Shanghai Jiao Tong University, Shanghai, P. R. China (corresponding author to provide e-mail: zhangaili@sjtu.edu.cn).

To date, most studies have been performed on the liposomalization of doxorubicin [10,11]. After injection, doxorubicin immediately disappears in blood [12], causing severe bone marrow suppression and cardiotoxicity [13]. Because doxorubicin has a strong anti-tumor effect and severe side toxicity, it is one of the typical drugs used for the drug delivery systems [14]. Compared to free doxorubicion, liposmal doxorubicin brings dramatic reduction in cardiac toxicity [15], reduces peak levels and sustains drug release into the blood stream [16]. Moreover, doxorubicin incorporated into long-circulating PEG-coated liposome demonstrates excellent effects in tumor therapy [17] and diminishes side effects, and is already used in clinical conditions.

Liposmal doxorubicin combined with hyperthermia can synergistically enhance anti-tumor cytotoxicity [18-20]. Previous studies on drug effect mainly focused on the tumor growth inhibition. To determine doxorubicin concentration in tumor, extracting method and HPLC are used [21, 22], as well as fluorescence spectrophotometry [23]. However, thru these methods, only the total amount of drug deposited in the tumor could be estimated but without any information of drug distribution in tumor. The thermally induced liposome nanoparticle extravasation has been found non-uniform and much more in the peripheral region than that in the tumor center [24], which would have different drug distributions in tumor and lead to different drug therapeutic efficacy.

In this study, we have chosen doxorubicin as anti-tumor drug model encapsulated by the PEG-coating liposome. Base on the ruby red self-fluorescence of doxorubicin and quantitave 3D method built thru our previous study [24], the dynamic process of the liposomal doxorubicin extravasation was quantitatively studied in different tumor regions using confocal fluorescence laser scanning microscopy. Moreover, the pathological analyses are performed of tumor tissues exercised on different days after the liposomal doxorubicin delivery combined with the local hyperthermia. Moreover, a simple diffusion model of liposomal doxorubicin to the tumor cells in vivo was used to well explain the experimental observations.

I. MATERIALS AND METHODS

A. Experimental study

Mice with tumor implantation in the dorsal skinfold window for 10 days were used. A total of 44 animals were divided into four groups (11 each) to study liposomal doxorubicin extravasation in the tumor center and periphery at 34°C and 42°C, respectively. The temperatures of the tumor

preparation and the water bath were monitored using thermocouples during the experiment.

In each experiment, the animals were anesthetized with ketamine-xylazine (5ul/g, *i.p.*). The 200*ul* suspension of the liposomal doxorubicin was injected into the tail vein. The scanning was performed every 10 minutes up to 1 hour after the injection of the liposomal doxorubicin. Once all collected, images at different depths of the tumor were studied to obtain the quantitative change of fluorescence intensity in the selected tissue volume with respect to time, which was directly related to the 3D extravasation of liposomal doxorubicin.

B. Liposomal doxorubicin preparation

Sterically stabilized long-circulating PEG liposome was prepared by the lipid film hydration and extrusion method. [25]. The final lipid concentration after hydration was 10mg/ml. The liposome size was determined by dynamic light scattering using a Zetasizer $3000HS_A$ (Malvern Instruments Ltd., U.K.). Liposomal doxorubicin was produced from doxorubicin hydrochloride with the pH gradient method [26].

C. Animal and tumor model

BALB/c nude mice (20±2g) were bought from the Animal Center, CAS, Shanghai, China. They were fed with sterile food, acidified water with the pH value kept at 2.5-2.8, and housed in the isolated cages with a 12-h light/dark cycle. The window chamber was surgically placed on the dorsal skin flap of the nude mouse [27], and a small volume ($0.1mm^3$) of murine mammary carcinoma 4T1 tissue was inoculated into the skin tissue within the window chamber.

D. Study of liposomal doxorubicin extravasation using laser confocal microscopy

Confocal laser scanning microscope LSM510 Meta (Zeiss, Germany) was used in this study. To image the liposomal doxorubicin distribution in tissue, argon laser (excitation: 488nm; emission: 520nm) was used. The tumor was viewed through the nude mice dorsal skin flap window using a 10X objective. Optical slicing was performed to collect 15-20 images throughout the tissue (approximately 150μm thick) every 10 minutes up to 1 hour after the liposomal doxorubicin injection. Extravasation of liposomal doxorubicin in the tumor center and the periphery was quantified under two conditions (34°C and 42°C) using our previous method [24].

E. Antitumor activity of liposomal Doxorubicin

Immediately after, or on 1st, 3rd, 6th day after the liposomal doxorubicin delivery combined with the local hyperthermia (42°C, 1h), the tumor tissues inside the dorsal skinfold window were exercised, fixed with 10% buffered formalin, and embedded with paraffin. The tissue slice was cut along a sagittal direction of skin tissue using Leica RM2126. Correlative histological sections of 5μm thickness were prepared and stained with hematoxylin and eosin. Tumor tissues in the tumor center and the periphery were investigated using a 100X oil objective. Images taken under the trans-illumination were collected.

II. RESULTS

A. Extravasation of liposomal doxorubicin in tumor tissue

. It was quite obvious that the liposomal doxorubicin extravascular accumulation increased after 1 hour at 42°C. More significantly, the liposomal doxorubicin extravasation was heterogeneous inside tumor. In the tumor periphery with active tumor angiogenesis, large amount of the liposomal doxorubicin extravasated into the tumor interstitium, while in other tumor regions with well developed vessels, the extravasation was much less.

Extravasation of 100nm liposomal doxorubicin further quantified using 3D fluorescence microscopic images. The results were shown in Figure 1.

The relative self-fluorescence intensity of doxorubicin in the tumor interstitium hardly changed at 34°C, but increased significantly by the local hyperthermia at 42°C. Further, the relative self-fluorescence intensity of doxorubicin in the tumor peripheral region was more than 2.3, as compared with 1.3 in the tumor center.

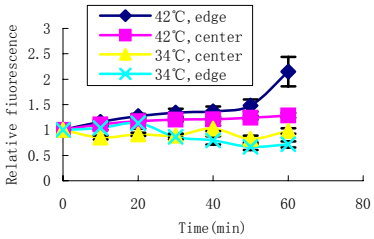
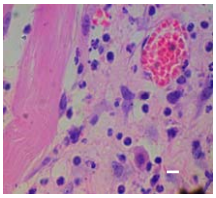


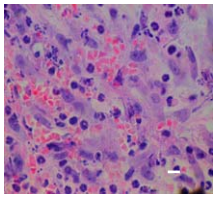
Figure 1 HT-induced extravasation of 100nm liposomal doxorubicin (DOX) at 42 ℃ or 34 ℃ for 1 hour in different tumor regions. The largest enhancement of extravasation was seen in the tumor periphery. Values are the mean and SE (n=11).

B. Histological analysis of antitumor activity of liposomal doxorubicin after hyperthermia

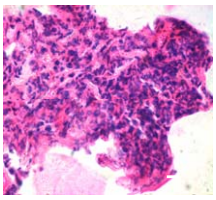
Immediately after, or on 1st, 3rd, 6th day after the liposomal doxorubicin delivery combined with the local hyperthermia (42°C, 1h), histological examination showed that there was no change found in both the tumor center and the peripheral region immediately after the treatment (Figure3 (a)-(b)); many nucleolus of tumor cells were condensed, collapsed, and disappeared in the peripheral region, but in the tumor center, there was no change found on the 1st day (Figure3 (c)-(d)); On the 3rd day, many tumor cells nucleolus began to condense, collapse (Figure3 (e)-(f)) in both regions; on the 6th day, both tumor cells in the peripheral and center region were found necrotic (Figure3 (g)-(h)).



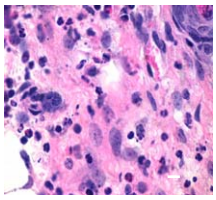
(a) the tumor center immediately after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr hour



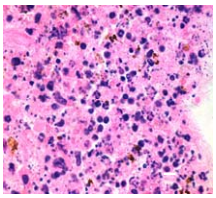
(b)the tumor periphery after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr



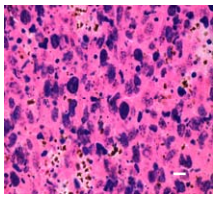
(c)the tumor center on the 1st day after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr



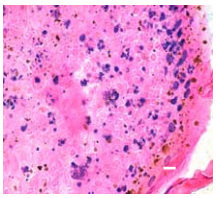
(d)the tumor periphery on 1st day after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr



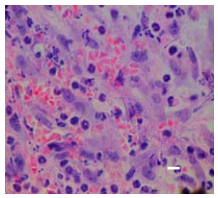
(e)the tumor center on the3rd day after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr



(f)the tumor periphery on the 3rd day after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr



(g)the tumor center on the 6th day after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr



(h)the tumor periphery on the 6th day after liposomal doxorubicin treatment combined with local hyperthermia at 42℃for 1hr

Figure 3 Drug effects of 100nm liposomal doxorubicin (DOX) treatment combined with hyperthermia at 42℃ for 1hr observed on the 1st, 3rd, 6th days after; viewed with 100X oil objective, Bar: 10μm.

III. THEORETICAL STUDY

According to the above study and a former paper from our group, the liposomes hardly extravasate in the central tumor region [24]. The radius of this region is about 60% of the tumor radius, which is around 1mm after 10 days. The 1mm tumor is thus divided to two cylindrical compartments: the central cylinder of a 0.6mm radius and the remaining annular peripheral region. The height of the tumor is about 0.15mm. The liposome extravasated in the periphery and free drug released from the liposomes will diffuse into the central region. Using a collective method, we have the following formular describing the liposome and free doxorubicin concentrations in the central compartment approximately,

$$D_{e,i}\cdot\frac{C_{p,i}-C_{c,i}}{r}\cdot A=\frac{dC_{c,i}}{dt}\cdot V \qquad (1)$$

Where $C_{c,i}$, $C_{p,i}$ are the concentration of liposomes or free drug in the central and peripheral region, respectively. $D_{e,i}$ is the diffusivity of the liposome or free drug in the tumor. A and V are the area of the interface between the central and peripheral compartments and volume of the tumor central compartment. The subscript *i* refers to the liposome or free Doxorubicin. Thus, the time constant of the diffusion, the time when the drug (liposome or free doxorubicin) concentration of the central compartments reaches ($1-\frac{1}{e}$), about 62.3% , of the peripheral region, is defined as,

$$\tau=\frac{V\cdot r}{D_{e,i}\cdot A} \qquad (2)$$

The diffusion of the free drug inside the tumor should mainly through the interstitial area [28]. The data was reported to be 1.2×10^{-8} cm^2/s.

The diffusivity of the liposome may be near to that of the macromolecules, whose diffusivity is about 1/10 of the small molecules in tumor [29]. Therefore the liposome diffusivity in tumor is assumed to be 1.2×10^{-8} cm^2/s.

By substituting the dimensional information and the diffusivity data into Eq.(2), the magnitude of the time constant to evaluate the drug delivery outcome could be obtained. The values for the liposome and the free dox are 400hours and 40 hours respectively. Besides, the liposome rupture time constant is about 12hours [30]. By comparing these data, we could conclude that after the thermal liposomal drug delivery, the diffusion of free drug released shall be the dominant and determines the therapeutic index. At the beginning of the delivery, most drug exists inside the liposomes and very few diffuse into the central region. The released free drug mainly accumulated in the tumor peripheral region and caused excessive necrosis of tumor cells in this region as shown in Fig.3c. Three days later, the liposomes have all ruptured, and as the time constant of the free Dox transport into the central region is about 2 days (40hours), a significant mass of drugs have reached the tumor central region. However as the therapeutic results of the cells depends not only on the drug concentration but also the time maintained at this concentration, or AUC, the area under the concentration-time curves, there is still difference between the peripheral and central regions as shown in Fig.3e and Fig.3f. However, for 6 days after treatment (much longer than 40hours), according to the theoretical analysis, adequate drug should have been delivered to the central region, and the tumor cells shall be destroyed. This explains the experimental results shown in Fig.3g and Fig.3h.

IV. Conclusion and Discussion

In this paper, real time 3D quantification of liposomal doxorubicin distribution in different tumor regions was quantified using confocal fluorescence microscopy.

The Results of the quantitative 3D study of the thermally induced 100nm liposomal doxorubicin extravasation in tumor was quite similar to that of the empty lipsome [24]. The liposomal doxorubicin extravasation was also non-uniform. The excessive liposomal doxorubicin extravasation mainly resulted from both the increased vascular wall permeability and vascular damage induced by hyperthermia in the peripheral region [24].

The drug effect of the thermal targeting drug delivery system was also investigated. The results showed that the nanoparticle anti-tumor drug carrier delivery could be significantly enhanced by the local hyperthermia and thermally targeted liposome anti-tumor drug delivery system be developed.

A theoretical analysis of the diffusion of liposomal doxorubicin showed that it took more than 40hrs for the doxorubicin to get into the tumor cells in the center region from the periphery region. The theoretical results could be used to well explain the experimental observations.

Considering the thermally induced liposomal doxorubicin extravasation is non-uniform, it seems quite difficult to kill all the tumor cells especially those in the tumor center region where the drug dose is lower. It is suggested that longer heating period be used to enhance the diffusion process.

Acknowledgment

This work has been supported by the Ministry of Science and Technology in China (2002CCA00600), the National Natural Science Funding of China (50506016) and Shanghai Science and Technology Commission of Shanghai Municipality(0452nm014).

References

[1] Torchilin VP. Drug targeting. Eur J Pharm Sci 2000; 11 Suppl 2:S81-91.

[2] Allen TM, Sapra P. Ligand-targeted liposomal anticancer drugs. Prog Lipid Res 2003; 42(5): 439-62.

[3] Woodle MC. Sterically stabilized liposome therapeutics. Adv Drug Deliv Rev 1995; 16: 249-265.

[4] Harashima H, Kiwada H. Liposomal targeting and drug delivery: Kinetic consideration. Adv Drug Rev 1996; 19: 425-444.

[5] Yuda T, Pongpaibul Y, Maruyama K, et al. Activity of amphipathic polyethylene glycol to prolong the circulation time of the liposomes. J Pharm Sci Jpn 1999; 59: 32-42.

[6] Torchilin VP, Omelyanenko VG, Papisov MI, et al. Poly(ethyleneglycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochem Biophys Acta 1994; 1195:11-20.

[7] Janzen J, Song X, Brooks DE. Interfacial thickness of liposomes containing Poly(ethyleneglycol)-cholesterol from electophoresis. Biophys J 1996; 70: 313-320.

[8] Sadzuka Y, Nakai S, Miyagishima A, et al. The effect of dose on the distribution of adriamycin encapsulated in polyethyleneglycol-coated liposomes. J Drug Target 1995; 3: 31-37.

[9] Maruyama K, Ishida O, Takizawa T, et al. Possibility of active targeting to tumor tissues with liposomes. Adv Drug Deliv Rev 1999; 40: 89-102.

[10] Gill PS, Espina BM, Muggia F, et al. Phase Ⅰ/Ⅱ clinial and pharmacokinetic evaluation of liposomal daunorubicin. J Clin Oncol 1995; 13: 996-1000.

[11] Allen TM, Hansen C, Martin F, et al. Liposomes containing synthetic lipid derivatives of polyethylene glycol show prolonged circulation half-lives in vivo. Biochim Biophys Acta 1991; 1066: 29-36.

[12] Eksborg S, Stendahl U, and Lonroth U. Comparative pharmacokinetic study of adriamycin and 4'-epi-adriamycin after their simultaneous intravenous administration. Eur J Clin Pharmacol 1986; 30: 629-631.

[13] Booser DJ, Hortobagyi GN. Anthracycline antibiotics in cancer therapy: Focus on drug resistance Drugs 1994; 47: 223-258.

[14] Yasuyuki S, Sadao H. Does the amount of an antitumor agent entrapped in liposomes influence its tissue distribution and cell uptake? Cancer Letters 1998; 131: 163-170.

[15] Safra T, Muggia F, Jeffers S, et al. Pegylated liposomal doxorubicin (doxil): reduced clinical cardiotoxicity in patients reaching or exceeding cumulative doses of 500 mg/m2. Ann Oncol 2000; 11(8): 1029-33.

[16] Bally MB, Nayar R, Masin D, et al. Liposomes with entrapped doxorubicin exhibit extended blood residence times. Biochim Biophys Acta 1990; 1023(1): 133-9.

[17] Gabizon AA.Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Res 1992; 52(4): 891-6.

[18] Ning S, Macleod K, Abra RM, *et al.*, Hyperthermia induces doxorubicin release from long-circulating liposomes and enhances their anti-tumor efficacy. *Int J Radiat Oncol Biol Phys* 1994; 29：827-834.

[19] Gaber MH, Wu NZ, Hong K, *et al*., Papahadjopoulos D, Thermosensitive liposomes: extravasation and release of contents in tumor microvascular networks. *Int J Radiat Oncol Biol* Phys 1996; 36：1177-1187.

[20] Maruyama K, Unezaki S, Takahashi N, *et al*., Enhanced delivery of doxorubicin to tumor by long-circulating thermosensitive liposomes and local hyperthermia. *Biochim Biophys Acta* 1993; 1149：209-216.

[21] Embree L, Gelmon KA, Lohr A, *et al*., Chromatohraphic analysis and pharmacokinetics of liposome-encapsulated doxorubicin in nonsmall cell lung cancer patients，*J. Pharm Sci* 1993; 82： 627-634.

[22] Nicholis G, Clark BJ, Brown JE, Solid-phase extraction and optimized separation of doxorubicin, epirubicin and their metabolites using reversed-phase high-performance liquid chromatography， *J. Pharmaceut Biomed Anal* 1992; 10：949-957.

[23] Sadzuka Y, Nakai S, Miyagishima A, *et al.*, The effect of dose on the distribution of adriamycin encapsulated in polyehyleneglycol-coated liposomes， *J Drug Targeting* 1995; 3：31-37.

[24] Liu, Ping, Zhang Aili, Xu, Yuhong Xu, Lisa X. Study of non-uniform nanoparticle liposome extravasation in tumor, International Journal of Hyperthermia 2005; 21（3）：259-270

[25] Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by a rapid extrusion procedure: Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta* 1985; 812:55-65.

[26] Mayer, L. D., Tai, L. C., Bally, M. B., Mitilenes, G. N., Ginsberg, R. S. and Cullis, P. R.: Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients. Biochim Biophys Acta 1990; 1025: 143.

[27] Huang Q, Shan S, Braun RD, Lanzen J, Anyarambhatla G, Kong G, *et al.* Noninvasive visualization of tumor in rodent dorsal skin window chambers. *Nat Biotechnol* 1999； 17: 1033-1035.

[28] Lankelma J, Luque RF, Dekker H, *et al*., A mathematical model of drug transport in breast cancer, *Micro. Res*., 2000, 59：149-161.

[29] Brown EB, Boucher Y, Nasser S, *et al*., Measurement of macromolecular diffusion coefficients in human tumors, *Micro. Res.*, 2004, 67：231-236.

[30] El-Kareh AW, Secomb TW. A mathematical model for comparison of bolus injection, continuous infusion, and liposomal delivery of doxorubicin to tumor cells. Neoplasia. 2000, 2(4): 325-38.