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Accelerated clearance of a second injection of PEGylated liposomes in mice

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Abstract

We recently reported that the firstly injected PEGylated liposomes dramatically affected the rate of blood clearance of secondly injected PEGylated liposomes in rats in a time interval of injection dependent manner [J. Control. Release (2003)]. Mice are frequently used in evaluations of the therapeutic efficacy of PEGylated liposomal formulations, but the pharmacokinetics of repeatedly injected PEGylated liposomes in mice is not fully understood. In this study, therefore, we examined in mice the effect of the repeated injection of PEGylated liposomes on their pharmacokinetics. An intravenous pretreatment with PEGylated liposomes produced a striking change in the biodistribution of the second dose which was given several days after the first injection. The first dose resulted in a reduction in the circulation half-life of the second dose. The degree of alteration was dependent on the time interval between the injections. The rapid clearance of the second dose was strongly related to hepatic clearance (CLh). This finding suggests that a considerable increase in hepatic accumulation accounts for this phenomenon. But, no liver injury or an increase in the number of Kupffer cells were detected in histopathological evaluations. Collectively, although the multiple injections of the PEGylated liposomes had no obvious physical effects, such as inflammation, their pharmacokinetic behavior was clearly altered in mice. The results obtained here have important implications not only with respect to the design and engineering of liposomes for human use, but for evaluating the therapeutic efficacy of liposomal formulations in experimental animal models as well.

Keywords: PEGylated liposomes; Repeated injection; Accelerated clearance; Laboratory examination; Polyethylene glycol (PEG)

1. Introduction

The rapid uptake of intravenously injected particulate drug carriers by mononuclear phagocyte system (MPS) cells is the main limiting factor in the efficient targeting of a drug to other sites in vivo (Klibanov et al., 1990). Polyethylene glycol (PEG) polymers are currently the most widely used materials to modify particulate surfaces in order to avoid recognition by MPS cells. The resulting PEG 'brush' confers to particles, such as liposomes, a long-circulating lifetime,

Abbreviations: AUC, area under the blood concentration–time curve; CHOL, cholesterol; ^{3}H-CHE, ^{3}H-cholesterylhexadecyl ether; CLh, hepatic clearance; CLs, splenic clearance; DXR, doxorubicin; HEPC, hydrogenated egg phosphatidylcholine; $mPEG_{2000}$-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; MPS, mononuclear phagocyte system; PEG, polyethylene glycol; PL, phospholipid

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resulting in the alteration of their pharmacokinetics, an increase in efficacy and a reduction in the toxicity of drugs associated with them (Allen and Hansen, 1991).

Clinical applications of PEGylated liposomal formulations frequently require multiple injections. In fact, the use of a repeated injection regimen for the treatment of several diseases has been extensively used for chemotherapeutic drug-containing PEGylated liposomes (Gordon et al., 2000; Halm et al., 2000; Hubert et al., 2000). It is therefore surprising that studies on the effect of PEGylated liposomes on the pharmacokinetic behavior of subsequently injected doses have not been reported for human subjects. It is noteworthy that only a few studies have been reported on rats (Oussoren and Storm, 1999; Dams et al., 2000; Ishida et al., 2003), rhesus monkeys (Dams et al., 2000) and rabbits (Goins et al., 1998). The results of these studies suggest that the intravenous administration of PEGylated liposomes significantly alters the pharmacokinetic behavior of subsequently injected PEGylated liposomes: the circulation half-life of the second dose is dramatically decreased in a time- and frequency-dependent manner.

Mice are frequently used in evaluations of the therapeutic efficacy of liposomal formulations that contain chemotherapeutic agents or antibiotic agents (Vanetten et al., 1995a, 1995b; Allen, 1997; Sparano and Winer, 2001), but the pharmacokinetics of repeatedly injected liposomes in mice is not fully understood. A remarkable alteration in pharmacokinetic behavior of subsequently injected PEGylated liposomes in mice, if it occurs, would result in changes in the in vivo behavior of liposome-associated drugs, presumably leading to a change in their therapeutic efficacy and an increase in their toxicity. Dams et al. (2000) recently reported, in a mice study, that the first dose of PEGylated liposomes had no effect on the pharmacokinetic behavior of the second dose. In their study, however, the mice received a dose at weekly intervals and, as a result, the time interval for injections might have been not appropriate for detecting the accelerated clearance of the second injected dose. Therefore, further experiments will be required to address the issue of whether the first dose of PEGylated liposomes have an effect on the pharmacokinetic behavior of the second dose in mice.

In this study, we report on a detailed study of the influence of the pre-injection of PEGylated liposomes and the time-interval dependency on the pharmacokinetics of the second dose in mice. The issue of whether the first dose of injected PEGylated liposomes causes liver injury was also investigated by histopathological evaluation. In addition, the species difference between mice and rats in occurrence of the accelerated blood clearance of secondly administered PEGylated liposomes was discussed.

2. Materials and methods

2.1. Materials

Hydrogenated egg phosphatidylcholine (HEPC) and 1,2-distearoyl-*sn*- glycero-3- phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by the Nippon Oil and Fat Co., Ltd. (Tokyo, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. ^{3}H-Cholesterylhexadecyl ether (^{3}H-CHE) and ^{3}H-inulin were purchased from NEN Research Products (MA, USA). Sepharose CL-4B was purchased from Amersham-Pharmacia Biotech (Upsala, Sweden). All other reagents were of analytical grade.

2.2. Preparation of liposomes

Small unilamellar vesicles were prepared as previously described (Lopes de Menezes et al., 1998). PEGylated liposomes were composed of HEPC:CHOL:$mPEG_{2000}$-DSPE (1.85:1:0.15, molar ratio) and sized by subsequent extrusion through polycarbonate membrane filters (Nuclepore, CA, USA) with pore sizes of 400, 200, 100 and 80 nm. The mean diameters of the liposomes were in the range of 90–100 nm, as determined by a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The concentration of phospholipid (PL) was determined by colorimetric assay (Bartlett, 1959). To determine the biodistribution of the second dose, the liposomes were labeled with a trace amount of ^{3}H-CHE or ^{3}H-inulin (40 μCi/μmol of PL) as a nonexchangeable lipid phase marker and an aqueous phase marker, respectively. Unencapsulated ^{3}H-inulin was removed by chromatography on a Sepharose CL-4B column eluted with HEPES buffered saline (pH 7.4) prior to injection.

2.3. Animal

Male Std:ddY mice weighing 20–23 g were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Faculty of Pharmaceutical Sciences, The University of Tokushima.

2.4. Biodistribution and pharmacokinetics of PEGylated liposomes

In the first administration, the mice received PEGylated liposomes (0.5 μmol PL/mouse, approximately 25.0 μmol PL/kg body weight) via the tail vein. To determine the biodistribution of the second dose of PEGylated liposomes, the mice were injected intravenously ^{3}H-CHE or ^{3}H-inulin labeled PEGylated liposomes (0.5 μmol PL/mouse) via the tail vein. At selected post-injection time points, the mice were sacrificed. A blood sample was then obtained by heart puncture, and the liver and spleen were collected from the mice after withdrawing the blood sample. Blood correction factors were applied to all organ samples (Allen, 1984). Mice that received only one injection of ^{3}H-CHE labeled PEGylated liposomes served as controls. Radioactivities in blood and tissues were assayed as described previously (Harashima et al., 1993a).

Pharmacokinetics were analyzed based on a two-compartment model using the least squares parameter estimation program PKAnalyst (Micromath, UT, USA) as follows:

$$\mathrm{Cb} = A\exp(-\alpha t) + B\exp(-\beta t) \quad (1)$$

The hepatic clearance (CLh) and splenic clearance (CLs) were calculated as follows:

$$\mathrm{CLh} = \frac{\mathrm{Xh}(24)}{\mathrm{AUC}(0\text{–}24)} \quad (2)$$

$$\mathrm{CLs} = \frac{\mathrm{Xs}(24)}{\mathrm{AUC}(0\text{–}24)} \quad (3)$$

where Xh(24) and Xs(24) are the hepatic uptake and splenic uptake of ^{3}H-CHE labeled PEGylated liposomes at 24 h post-injection. AUC(0–24) is defined as the area under the blood concentration–time curve (AUC) from time 0 to 24 h. It is assumed that the degree of CLh and CLs directly reflect the degree of active liposome uptake by the liver and spleen, respectively.

2.5. Histopathological evaluations

Mice were treated with a single intravenous injection of PEGylated liposomes at a dose of 0.5 μmol PL/mouse (approximately 25.0 μmol PL/kg body weight). These mice were sacrificed at 10-day post-injection, and the liver and spleen were collected. These tissues were fixed in 20% neutral buffered formalin. After embedding in paraffin, the organs were sectioned at 5 μm, and stained with hematoxylin and eosin. Histopathological changes in the tissues were then evaluated. In addition, the number of total cells and sinusoidal wall cells in randomly selected areas of the liver were counted by an investigator (K.I.), who was blind with respect to the experimental groups, and the density of sinusoidal wall cells to total cells (%) was calculated. Mice that did not receive PEGylated liposomes served as controls.

2.6. Statistics

Statistical analyses (Student's *t*-test) were performed using StatView software (Abacus Concepts, Inc., CA, USA). A probability value of <0.05 was considered to indicate a significant difference.

3. Results

Blood clearance profiles for the second dose of PEGylated liposomes are shown in Fig. 1. The profile in the absence of a prior injection is also shown as a control (AUC(0–24) = 485.6 ± 38.1%dose·h/ml, $t_{1/2\beta}$ = 12.9 ± 2.8 h). At 5 or 7 days post-injection, no significant changes in the blood clearance profile were observed compared to the controls (AUC(0–24) for 5 days = 397.7 ± 59.1%dose·h/ml ($P = 0.0962$), for 7 days = 337.8 ± 91.8%dose·h/ml ($P = 0.0617$)). At 10 days post-injection, the second dose was cleared rapidly from the circulation compared to the control (AUC(0–24) = 221.2 ± 93.1 %dose·h/ml ($P < 0.05$), $t_{1/2\beta}$ = 6.3 ± 3.4 h ($P < 0.01$)). When the time interval for injection was extended to 14 days, the pharmacokinetics of the second dose returned to a profile similar to the controls (AUC(0–24) = 458.7 ± 49.4%dose·h/ml, $P = 0.4974$).

Time courses for the accumulation of the second dose in the liver and spleen were also investigated. In

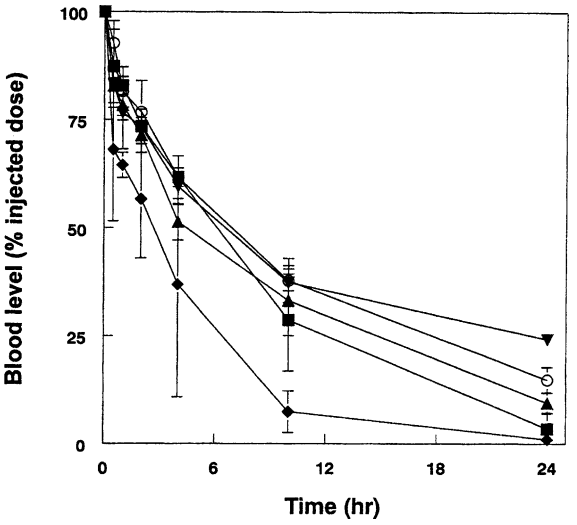

Fig. 1. Blood clearance profile of PEGylated liposomes in mice. Control (first dose) (○), second dose at 5 days (▲), 7 days (■), 10 days (◆), and 14 days (▼) post-injection. Mice that received only one injection of ^{3}H-CHE labeled PEGylated liposomes served as control. Each value represents the mean ± S.D. of three separate experiments.

the absence of the prior dose (control), the liposomes showed a lower accumulation in liver (Fig. 2). A highly increased hepatic uptake at 24 h was observed at 7 and 10 days post-injection. Interestingly, at 10 days post-injection, rapid hepatic accumulation was observed: the accumulation dramatically increased even 30 min after the injection of the second dose, and further increased gradually, reaching a maximum level at 10 h. The increased hepatic accumulation was normalized up to 14 days post-injection. In the spleen, lower accumulation levels were observed (less than 5% of the injected dose) and the splenic accumulation of the second dose was much lower relative to their hepatic accumulation in the entire course of this study (data not shown).

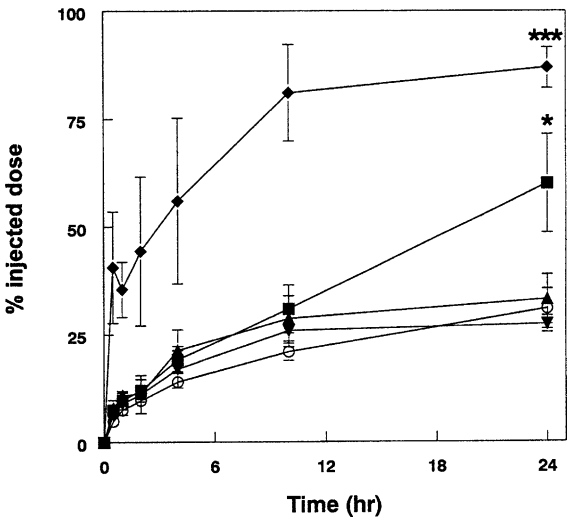

Fig. 2. Time courses of hepatic accumulations of PEGylated liposomes in mice. Each value represents the mean ± S.D. of three separate experiments.

CLh and CLs are shown in Fig. 3. These pharmacokinetic parameters reflect degree of activity of PEGylated liposome taken up by the liver and spleen, respectively. Significant increases in both CLh and CLs were observed in a time interval of injection dependent manner. With respect to CLh, the value increased 2.7- and 7.8-fold at 7 and 10 days post-injection compared to that for the control, respectively. Concerning the CLs, the values were much lower relative to CLh, increasing by 1.8- and 3.1-fold at 7 and 10 days post-injection, respectively (Fig. 3, inserted figure). These findings clearly indicate that the unexpected change in pharmacokinetic behavior of the second dose is due to the highly increased active uptake by liver and spleen, but that the contribution of spleen is much lower relative to that of liver.

In non-vehicle treated mice (normal mice), the blood clearance profile of ^{3}H-inulin-containing PEGylated liposomes was similar to that of ^{3}H-CHE labeled liposomes (AUC(0–24) = 466.7 ± 7.4%dose·h/ml, $t_{1/2\beta}$ = 14.8 ± 4.1 h) (Fig. 4A). Low hepatic accumulation was observed (Fig. 4B). In mice that received a prior dose (10 days post-injection), the second dose rapidly disappeared from the blood circulation (AUC(0–24) = 182.2 ± 25.2%dose·h/ml, $t_{1/2\beta}$ = 6.0 ± 2.3 h) and the blood clearance profile was similar to that of ^{3}H-CHE labeled liposomes (Fig. 4A ($P = 0.5271$)). It should be noted with respect to hepatic accumulation that in the mice that received prior dose, a much lower accumulation was observed in the case where inulin was used as a liposome-marker compared to the CHE ($P < 0.05$) (Fig. 4B). In the course of this experiment, no significant differences in splenic accumulation were detected (data not shown).

In a series of evaluations, the animals tolerated the repeated injection of PEGylated liposomes with no change in body weight or general condition (data

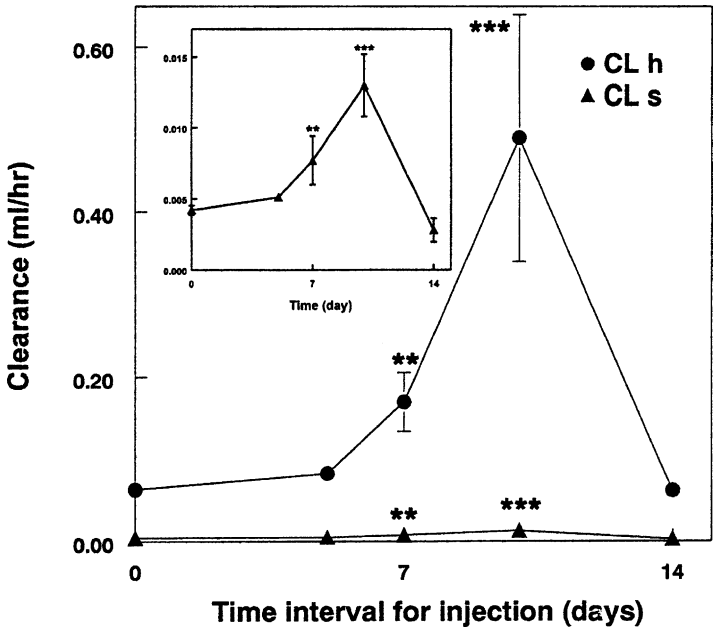

Fig. 3. Hepatic clearance (CLh) and splenic clearance (CLs) for the first or second dose of PEGylated liposomes in mice. CLh and CLs were analyzes on the basis of the data from Figs. 1 and 2 as described in Section 2.4. Each value represents the mean ± S.D. *P*-values apply to differences between the vehicle non-treated mice and vehicle-treated mice. $^{**}P < 0.01$, $^{***}P < 0.005$.

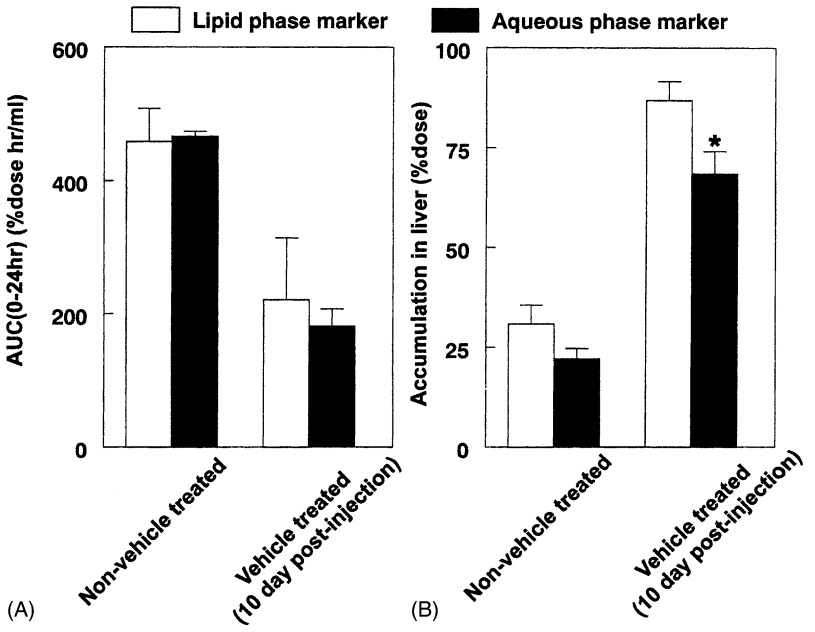

Fig. 4. (A) AUC for either ^{3}H-CHE or ^{3}H-inulin labeled PEGylated liposomes in either vehicle non-treated or vehicle-treated (10 days post-injection) mice. (B) Hepatic accumulation either ^{3}H-CHE or ^{3}H-inulin labeled PEGylated liposomes in mice either vehicle non-treated (normal mice) or received prior dose (10 days post-injection), at 24 h following administration. Each value represents the mean ± S.D. *P*-value applies to differences between the lipid phase marker (CHE) and aqueous phase marker (inulin). $^{*}P < 0.05$.

not shown). In addition, no significant change was detected in the percentage of liver weight of body weight (data not shown). A histopathological examination of slides of liver and spleen collected from the animal that received the PEGylated liposomes once were prepared and then compared to the slides prepared from non-vehicle treated mice (normal mice). Under our experimental condition, no histopathological changes (liver and spleen) were observed (data not shown). In addition, the number of sinusoidal wall cells per section of liver was counted by an investigator (K.I.), who was blind with respect to the experimental groups, and the density of the sinusoidal wall cells to total cells (%) in the liver was calculated. The density of the sinusoidal wall cells was 33.6 ± 4.0 (control; non-vehicle treated (normal)) and 31.8 ± 1.8 (10 days post-injection), respectively. No significant increase in the number of sinusoidal wall cells was observed in any of the liver slides ($P = 0.4959$), suggesting that the first injected dose did not increase the number of Kupffer cells in the liver.

4. Discussion

The present study demonstrates a remarkable change in the pharmacokinetic behavior and the hepatic accumulation of a second injection of PEGylated liposomes in mice. Our finding in the present study is inconsistent to that of Dams et al. (2000). This discrepancy could be due to differences in experimental designs: they injected PEGylated liposomes once per week. Therefore, they might fail to detect the phenomenon observed in this study.

The accelerated clearance for the second dose is strongly related to the increased CLh levels (Figs. 1 and 3), suggest that liver cells, presumably Kupffer cells, play an important role in the effect observed. However, no significant abnormalities or an increase in the number of Kupffer cells in the liver were detectable in any of the histopathological evaluations. This suggests that the first dose may have given the liver a mild stimulation, resulting in neither liver injury nor an increase in the number of Kupffer cells at 10 days post-injection. Dams et al. (2000), using a rat system, proposed that a transfusable serum factor was responsible for the accelerated clearance of the second dose of PEGylated liposomes. Most recently, Laverman et al. (2001) proposed that macrophages that have taken up the first dose were responsible for the production of the serum factor in rats. Collectively, these findings lead to an assumption that the stimulation of the liver by the first dose induces the production and excretion of the serum factor, and the second dose that then opsonizes with the factor are recognized by the macrophages in the liver, resulting in the rapid clearance from the circulation (Fig. 1) and a rapid accumulation in the liver (Fig. 2) in mice.

It is well-known that the destabilization of liposomes and resulting release of their contents is one of the dominant processes that determines the clearance rate and tissue distribution of intravenously injected liposomes (Panagi et al., 1998; Ishida et al., 2000). We employed inulin as an aqueous phase marker, instead of CHE, a lipid phase marker, to evaluate whether enhanced liposome destabilization also occurs in parallel with the accelerated clearance phenomenon. Inulin, once released from the liposomes, is never distributed to phagocytic cells and is rapidly excreted by the kidney via glomerular filtration (Harashima et al., 1993b). The results indicate that approximately 18% of the injected liposomes released their contents at 24 h under the accelerated clearance phenomenon (Fig. 4). This finding clearly shows that the accelerated clearance of the second dose is the result of a combination of increased accumulation in liver and destabilization in the blood if liposome-associated drugs which are encapsulated into an aqueous phase are used, although hepatic accumulation is the main contributor.

We recently reported that the first dose of PEGylated liposomes resulted in a reduction in the circulation time and an increase in hepatic accumulation of the second dose in rats (Ishida et al., 2003). This report clearly indicates that similar phenomenon occurs in both animals, rats and mice, when the PEGylated liposomes were injected repeatedly. It is noted that the observed phenomenon was found especially when the time interval following the first injection was approximately 5 days in rats but approximately 10 days in mice. This difference indicates that there is species difference in the rate of effectuation of the phenomenon among rats and mice: the accelerated clearance effect is induced more rapidly in rats than in mice under the conditions used in our experiments. Accounting for the reason for the species difference is not an easy task, since the underlying mechanism for the effectuation

of the phenomenon is not clear at this stage. However, these observations at least lead an assumption that similar phenomenon possibly occurs in human when the PEGylated liposomes are injected repeatedly. The pharmacokinetic change would result in an alteration of the in vivo behavior of liposome-associated drugs; the shortened circulation-time compromises the therapeutic efficacy of liposome-associated drugs and the considerably increased hepatic accumulation may increase their toxicity in liver, leading to, for example, fulminant hepatic necrosis (Daemen et al., 1995), if the injected liposomes contain cytotoxic agents. In fact, Hengge et al. (1993) reported on an AIDS patient with Kaposi's sarcoma who received biweekly cycles of PEGylated liposomal doxorubicin (DXR) and died 14 weeks after liposomal DXR administration from hepatorenal failure. Accordingly, there would be a frequently need to tailor optimal dosage regimen of liposomal formulation to patient. A failure to do so can lead to ineffective therapy or toxicity to patients.

The physicochemical properties of liposomes such as PEGylation, the dose used for injection, and related issues, may be important factors in causing the pharmacokinetic change when the PEGylated liposomes are repeatedly injected to human, although the underlying mechanism is currently unclear. Therefore, further systemic investigations will be required to predict if the accelerated clearance occurs and to determine the optimal dosage regimen with respect to PEGylated liposomal formulations in human. Based on our present and recent results, we conclude that it might be possible to avoid the occurrence of the accelerated clearance phenomenon in human by selecting and adjusting the time interval between injections. As shown in Fig. 1, in mice study, an accelerated clearance for the second dose was not observed when the time interval was reduced to 7 days and was attenuated when the interval was extended to 14 days. In rats study, we reported that an accelerated clearance for the second dose did not occur when the time interval was reduced to 3 days or extended to 4 weeks (Ishida et al., 2003). Several studies have reported importance of the dosing schedule on the pharmacokinetics of serial injections of PEGylated liposomes. Goins et al. (1998) observed similar pharmacokinetics for subsequently injected PEGylated liposomes in rabbits when given in a 6-week interval. Oussoren and Storm (1999) demonstrated that there was no effect on the circulation kinetics when PEGylated liposomes were given to rats at 24 or 48 h dosing intervals. These findings would strongly support our conclusion. In fact, the low injection frequency of PEGylated liposomal DXR (3–6 weeks) have been chosen to prevent the occurrence of cutaneous toxicity in clinical settings (Muggia et al., 1997).

Liposomes represent one of the promising carriers for improving the therapeutic index of associated therapeutic molecules such as drugs, proteins and DNA, by increasing their localization to specific target tissues or cells and by decreasing their localization to normal tissues. In clinical settings, the repeated injection of therapeutic molecules-containing liposomes can not be excluded. Furthermore, mice are frequently used for evaluating the therapeutic efficacy of liposomal formulations and other particulate carrier systems. Hence, the results demonstrated here could be of importance in the future use, design and engineering of long-circulating carrier systems for human use, and could have important implications with respect to the evaluation of the therapeutic efficacy of liposomal formulations in animal models.

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References

Allen, T.M., 1984. Stealth liposomes: avoiding reticuloendothelial uptake. In: Lopez-Berestein, G., Fidler, I. (Eds.), Liposomes in the Therapy of Infectious Disease and Cancer, vol. 3. UCLA Symposium in Molecular and Cellular Biology. CRC Press, Boca Raton, FL, pp. 205–224.

Allen, T.M., 1997. Liposome targeting in animal models: problems and opportunities. J. Liposome Res. 7, 315–329.

Allen, T.M., Hansen, C.B., 1991. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim. Biophys. Acta 1068, 133–141.

Bartlett, G.R., 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234, 466–468.

Daemen, T., Hofstede, G., Ten Kate, M.T., Bakker-Woudenberg, I.A., Scherphof, G.L., 1995. Liposomal doxorubicin-induced toxicity: depletion and impairment of phagocytic activity of liver macrophages. Int. J. Cancer 61, 716–721.

Dams, E.T.M., Laverman, P., Oyen, W.J.G., Storm, G., Scherphof, G.L., Van der Meer, J.W.M., Corstens, F.H.M., Boerman, O.C., 2000. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. J. Pharmacol. Exp. Ther. 292, 1071–1079.

Goins, B., Phillips, W.T., Klippers, R., 1998. Repeat injection studies of technetium-99m-labeled PEG-liposomes in the same animal. J. Liposome Res. 8, 265–281.

Gordon, A.N., Granai, C.O., Rose, P.G., Hainsworth, J., Lopez, A., Weissman, C., Rosales, R., Sharpington, T., 2000. Phase II study of liposomal doxorubicin in platinum- and paclitaxel-refractory epithelial ovarian cancer. J. Clin. Oncol. 18, 3093–3100.

Halm, U., Etzrodt, G., Schiefke, I., Schmidt, F., Witzigmann, H., Mossner, J., Berr, F., 2000. A phase II study of pegylated liposomal doxorubicin for treatment of advanced hepatocellular carcinoma. Ann. Oncol. 11, 113–114.

Harashima, H., Yamane, C., Kume, Y., Kiwada, H., 1993a. Kinetic analysis of AUC-dependent saturable clearance of liposomes: mathematical description of AUC dependency. J. Pharm. Biopharm. 21, 299–308.

Harashima, H., Kume, Y., Yamane, C., Kiwada, H., 1993b. Kinetic modelling of liposome degradation in blood circulation. Biopharm. Drug Dispos. 14, 265–270.

Hengge, U.R., Brockmeyer, N.H., Rasshofer, R., Goos, M., 1993. Fatal hepatic failure with liposomal doxorubicin. Lancet 341, 383–384.

Hubert, A., Lyass, O., Pode, D., Gabison, A., 2000. Doxil (caelyx): an exploratory study with pharmacokinetics in patients with hormone-refractory prostate cancer. Anticancer Drugs 11, 123–127.

Ishida, T., Kojima, H., Harashima, H., Kiwada, H., 2000. Biodistribution of liposomes and C3 fragments associated with liposomes: evaluation of their relationship. Int. J. Pharm. 205, 183–193.

Ishida, T., Maeda, R., Ichihara, M., Irimura, K., Kiwada, H., 2003. Accelerated clearance of PEGylated liposomes in rat after repeated injection. J. Control. Release 88, 35–42.

Klibanov, A.L., Maruyama, K., Torchilin, V.P., Huang, L., 1990. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett. 268, 235–237.

Laverman, P., Carstens, M.G., Boerman, O.C., Dams, E.T.M., Oyen, W.J.G., Rooijen, N.V., Corstens, F.H.M., Storm, G., 2001. Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. J. Pharmacol. Exp. Ther. 298, 607–612.

Lopes de Menezes, D.E., Pilarski, L.M., Allen, T.M., 1998. In vitro and in vivo targeting of immunoliposomal doxorubicin to human B-cell lymphoma. Cancer Res. 58, 3320–3330.

Muggia, F., Hainsworth, J.D., Jeffers, S., Miller, P., Groshen, S., Tan, M., Roman, L., Uziely, B., Muderspach, L., Garcia, A., Burnett, A., Greco, F.A., Morrow, C.P., Paradiso, L.J., Liang, L.-J., 1997. Phase II study of liposomal doxorubicin in refractory ovarian cancer: antitumor activity and toxicity modification by liposomal encapsulation. J. Clin. Oncol. 15, 987–993.

Oussoren, C., Storm, G., 1999. Effect of repeated intravenous administration on the circulation kinetics of poly(ethyleneglycol)-liposomes in rats. J. Liposome Res. 9, 349–355.

Panagi, Z., Avgoustakis, K., Evangelators, G., Ithakissios, D.S., 1998. Protein-induced CF release from liposomes in vitro and its correlation with the BLOOD/RES biodistribution of liposomes. Int. J. Pharm. 163, 103–114.

Sparano, J.A., Winer, E.P., 2001. Liposomal anthracyclins for breast cancer. Semin. Oncol. 4 (Suppl. 12), 32–40.

Vanetten, E.W.M., Vanvianen, W., Tijhuis, R.H.G., Storm, G., Bakkerwoudenberg, I.A.J.M., 1995a. Sterically stabilized amphotericin B liposomes: toxicity and biodistribution in mice. J. Control. Release 37, 123–129.

Vanetten, E.W.M., Ottelambillion, M., Vanvianen, W., Tenkate, M.T., Bakkerwoudenberg, I.A.J.M., 1995b. Biodistribution of liposomal amphotericin B (ambisome) and amphotericin B desoxycholate (fungizone) in uninfected immunocompetent mice and leucopenic mice infected with *Candida albicans*. J. Antimicrob. Chemother. 35, 509–519.