**Table 3.** The Pharmacokinetic Parameters of Inner ADP [(8C–$^{14}$C)ADP] and Outer Lipids Membranes [(1,2-$^{3}$H(N)-cholesterol)] Derived from 3H, $^{14}$C Radiolabeled H12-(ADP)-Liposomes After an Intravenous Injection at a Dose of 10 mg Lipids/kg to Normal Rats and Thrombocytopenic Rats

| | Normal Rats | | Thrombocytopenic Rats | |
|---|---|---|---|---|
| | $^{3}$H | $^{14}$C | $^{3}$H | $^{14}$C |
| $t_{1/2}$ (h) | 8.18 ± 0.77 | 8.21 ± 1.01 | 1.81 ± 0.39** | 1.68 ± 0.78** |
| MRT (h) | 10.2 ± 1.18 | 10.4 ± 1.46 | 2.17 ± 0.71** | 2.09 ± 1.01** |
| AUC (h % of dose/mL) | 58.4 ± 6.5 | 54.2 ± 10.1 | 10.7 ± 4.1** | 10.2 ± 4.3** |
| CL (mL/h) | 1.73 ± 0.18 | 1.89 ± 0.32 | 10.4 ± 4.2** | 11.4 ± 5.4** |
| $V_{dss}$ (mL) | 17.7 ± 3.5 | 19.5 ± 3.6 | 20.7 ± 2.9 | 20.2 ± 1.9 |

The data for the normal rats were cited from our previously reported paper.[17]
**$p < 0.01$ versus normal rats.
Each value represents the mean ± SD ($n = 4$).
$t_{1/2}$, half-life; MRT, mean residence time; AUC, area under the concentration–time curve; CL, clearance; $V_{dss}$, distribution volume.

was approximately four times shorter than that in normal rats ($^{14}$C; 8.21 ± 1.01 and 1.68 ± 0.78 h, $^{3}$H; 8.18 ± 0.77 and 1.81 ± 0.39 h, for normal and thrombocytopenic rats, respectively). In addition, CL in thrombocytopenic rats was increased compared with normal rats ($^{14}$C; 1.89 ± 0.32 and 11.4 ± 5.4 mL/h, $^{3}$H; 1.73 ± 0.18 and 10.4 ± 4.2 mL/h, for normal and thrombocytopenic rats, respectively), and the AUC was decreased in the case of the thrombocytopenic rats compared with the normal rats ($^{14}$C; 54.2 ± 10.1 and 10.2 ± 4.3 h % of dose/mL, $^{3}$H; 58.4 ± 6.5 and 10.7 ± 4.1 h % of dose/mL, for normal and thrombocytopenic rats, respectively). Interestingly, although the plasma concentration curves for $^{14}$C, $^{3}$H radiolabeled H12-(ADP)-liposome exhibited similar behaviors for up to 24 h after injection in normal rats,[17] indicating that the H12-(ADP)-liposomes circulate in the bloodstream without any leakage of ADP, the plasma concentration curves of $^{14}$C radioactivity and $^{3}$H radioactivity and pharmacokinetic parameters in thrombocytopenic rats were different. However, previous *in vivo* hemostatic studies of H12-(ADP)-liposomes clearly showed that the tail vein bleeding times for busulphan-induced thrombocytopenic rats after an infusion of H12-(ADP)-liposomes (10 mg lipids/kg) were significantly reduced compared with that of nontreatment rats.[14,16] These results indicate that the H12-(ADP)-liposomes likely circulate in the bloodstream in a form available for exerting hemostasis until they reach the site of a vascular injury, and successfully augment hematostatic effects in thrombocytopenic rats.

### *Tissue Distribution*

Liposomes are generally captured and degraded by the mononuclear phagocyte system (MPS) in the liver and spleen, such as by Kupffer cells and splenic macrophages.[25] As expected, both the $^{14}$C radioactivity and $^{3}$H radioactivity of the H12-(ADP)-liposomes were mainly distributed in the liver and spleen in thrombocytopenic rats at 2, 6, and 24 h after the administration of $^{14}$C, $^{3}$H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg (Fig. 3), but were scarcely distributed in kidney, lung, and heart (data not shown). This result is in general agreement with results reported in a previous study involving similar pharmacokinetic studies in normal rats.[17] A previous *in vivo* and *in vitro* finding reported that HbV, which has similar characteristics in terms of liposomal structure to H12-(ADP)-liposomes, was mainly distributed to the liver and spleen, and the specific uptake and degradation of HbV was observed only in macrophage cells (RAW 264.7 cells) but not in parenchymal and endothelial cells in the liver.[26] These results strongly suggest that the majority of the H12-(ADP)-liposomes are also scavenged and degraded by the MPS, likely by Kupffer cells or splenic macrophages, in thrombocytopenic rats.

As the liver and spleen are the major distribution tissues for H12-(ADP)-liposomes in both normal and thrombocytopenic rats, the hepatic and splenic distribution of H12-(ADP)-liposomes was compared. Figure 3 provides information on the tissue distribution in liver and spleen at 2, 6, and 24 h after the administration of $^{14}$C, $^{3}$H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg to normal and thrombocytopenic rats. The hepatic distributions of both $^{14}$C radioactivity and $^{3}$H radioactivity in thrombocytopenic rats were higher than that in normal rats (Figs. 3a and 3b). In a previous study, Telgenhoff et al.[27] reported that the greatest number of activated macrophages in the liver were observed in rats treated with the anticancer drug, cisplatin, as evidenced by light and electron microscopic analyses. In addition, the disease conditions including thrombocytopenia that induced cytokines productions may enhance the phagocyte activity of macrophages. Therefore, the possibilities that either/both busulphan or/and disease conditions such as thrombocytopenia may influence to the phagocyte activity of Kupffer cells and other type of macrophages (splenic macrophages and bone marrow macrophages etc.) leading to a change in the tissue distribution of H12-(ADP)-liposomes in busulphan-induced thrombocytopenic rats cannot be excluded. Further study will be necessary to elucidate whether the treatment with anticancer drugs or thrombocytopenic condition influence the macrophage activity from the viewpoint of future clinical applications of H12-(ADP)-liposomes in patients with anticancer drugs induced thrombocytopenia. On the contrary, the distribution of both $^{14}$C radioactivity and $^{3}$H radioactivity in the spleen was decreased in thrombocytopenic rats compared with that in normal rats (Figs. 3c and 3d). In addition, no difference was found in the tissue distribution of both the $^{14}$C radioactivity and $^{3}$H radioactivity of the H12-(ADP)-liposomes in other organs (kidney, lung, and heart) between normal and thrombocytopenia rats (data not shown). These data indicate that the H12-(ADP)-liposomes are mainly distributed to the liver and spleen in thrombocytopenic rats, and that the tissue distribution in the liver and spleen was changed when thrombocytopenia was present.

To develop an H12-(ADP)-liposome preparation for use as a platelet alternative, it is important to have an extensive knowledge of the characteristics of the biodegradable properties of

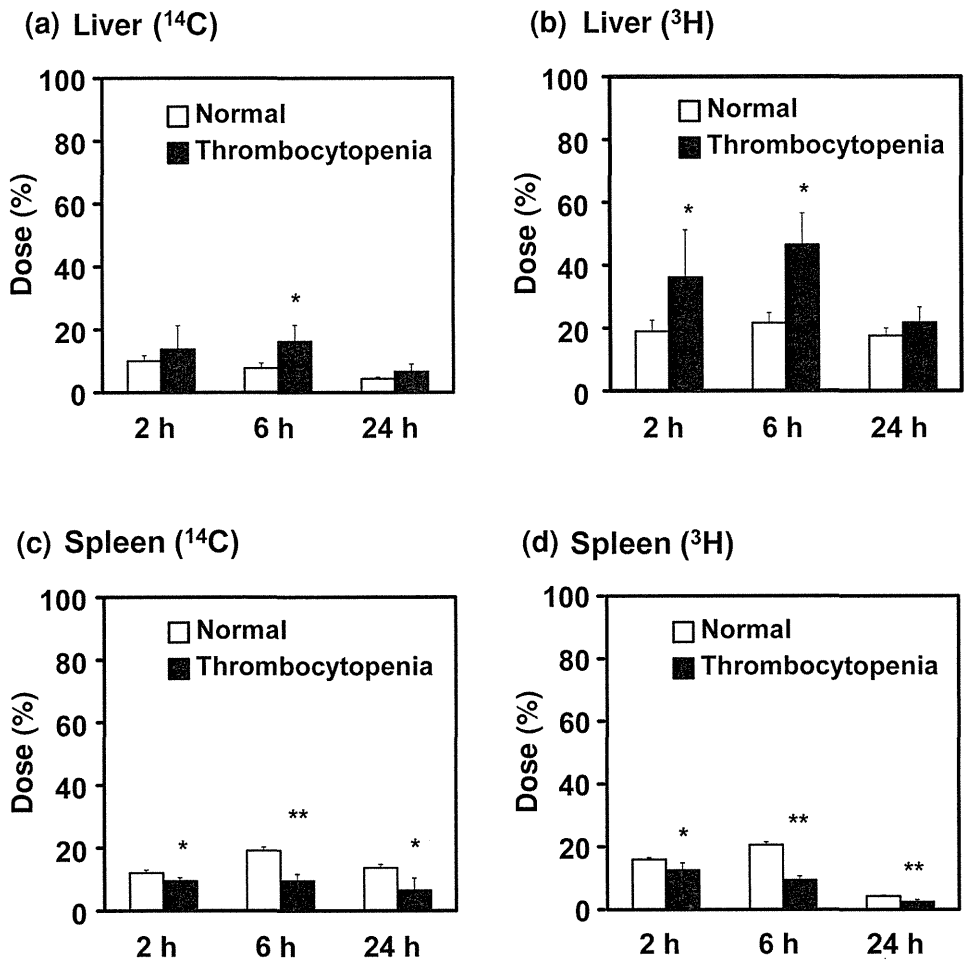


**Figure 3.** The hepatic distribution of (a) $^{14}$C radioactivity and (b) 3H radioactivity and splenic distribution of (c) $^{14}$C radioactivity and (d) 3H radioactivity at 2, 6, and 24 h after an intravenous injection of 3H, $^{14}$C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to normal rats (open bar) and thrombocytopenic rats (closed bar). The data for the normal rats were cited from our previously reported paper.[17] Each bar represents the mean ± SD ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus normal rats.

the compound (no accumulation or retention in tissues). In the present study, both the $^{14}$C radioactivity and $^{3}$H radioactivity of the H12-(ADP)-liposomes were eliminated from the bloodstream and organs, and the activity essentially disappeared within 7 days after injection of the H12-(ADP)-liposomes (data not shown), indicating that H12-(ADP)-liposomes and components derived from them do not accumulate in the body to any extent. Therefore, H12-(ADP)-liposomes contain the appropriate components and have the potential for use as a synthetic platelet substitute, because they possess acceptable biodegradable properties, even under conditions of thrombocytopenia.

### *Excretion*

In order to identify the excretion pathway of the H12-(ADP)-liposomes in thrombocytopenic rats, the levels of $^{14}$C and $^{3}$H in urine and feces were measured (Fig. 4). The $^{14}$C was excreted mainly in the urine (Fig. 4a; 85.7 ± 3.6% of the injected dose at 5 days after injection), but the levels were low in feces (Fig. 4c; 14.5 ± 6.9% of the injected dose at 5 day after injection). We previously showed that most of the encapsulated ADP in the H12-(ADP)-liposomes was mainly metabolized to allantoin, which is the final metabolite of ADP in rodents,[28] and was excreted into the urine within 7 days after the injection in normal rats.[17] Therefore, the ADP encapsulated within H12-liposomes was completely metabolized and excreted into the urine, even though ADP was encapsulated within the liposome, in thrombocytopenic rats. Furthermore, the enhanced excretion of $^{14}$C of ADP in feces was observed in thrombocytopenic rats as compared with normal rats (Fig. 4c). Although it is difficult to explain these unexpected results, these phenomena suggested that a part of encapsulated ADP and its metabolites in H12-(ADP)-liposomes are eliminated by bile excretion pathway, and it increases in thrombocytopenia. To clarify whether it is specific to busulphan, it will be necessary to examine the same experiments under the similar thrombocytopenia conditions induced by other anticancer agents.

On the contrary, the majority of the $^{3}$H was excreted in the feces (Fig. 4d; 70.8 ± 7.9% of the injected dose at 5 days after injection), and excretion into the urine was essentially nil (Fig. 4b). This result is in agreement with the disposition of H12-(ADP)-liposome in normal rats[17] and HbV that uses an HbV-labeled outer membrane with [$^{3}$H] cholesterol.[26] A previous report showed that cholesterol in liposomes reappears in the blood mainly as lipoprotein–cholesterol complexes after entrapment in Kupffer cells and should then be excreted via the bile after entrapment of the lipoprotein cholesterol com-

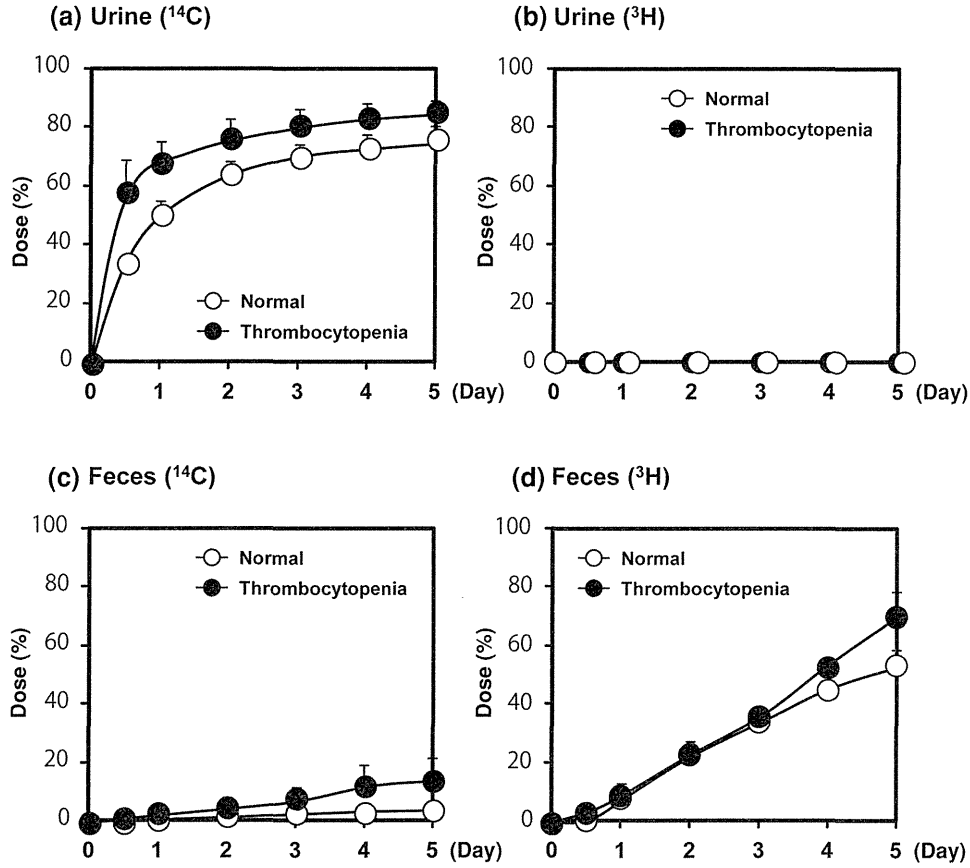


**Figure 4.** Time course for radioactivity in urine and feces after the administration of $^{3}$H, $^{14}$C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to normal rats (open circle) and thrombocytopenic rats (closed circle). The data for the normal rats were cited from our previously reported paper.[17] Each point represents the mean ± SD ($n$ = 4).

plex by hepatocytes.[29] Therefore, under conditions of thrombocytopenia, it is possible that the cholesterol in the H12-(ADP)-liposome is metabolized and excreted in the same manner as the other liposome components, as reported by Kuipers et al.[29] In fact, the amount of fecal excretion of $^{3}$H at 5 days after injection in thrombocytopenic rats was higher than that in normal rats (Fig. 4d) with the increment of hepatic distribution (Fig. 3b).

These results indicate that, even though the H12-(ADP)-liposomes and its components retained their desirable excretion properties in thrombocytopenic rats, the excretion pathway of the H12-(ADP)-liposomes components in thrombocytopenic rats were the same as that in normal rats.

## CONCLUSIONS

Our objective was to develop H12-(ADP)-liposome that possess the characteristics of, not only better pharmacological effects (suitable blood retention), but also acceptable biodegradable properties (no retention in the body), and to document these characteristics in, not only normal, but also under conditions of thrombocytopenia. In the present study, we provide the first demonstration of the preparation and function of H12-(ADP)-liposomes and show that that the disposition of H12-(ADP)-liposomes and components derived from them in thrombocytopenic rats induced by busulphan are as follows. After the systemic circulation of the H12-(ADP)-liposomes in the blood stream, they were mainly distributed to the liver and spleen, where they are degraded by the MPS. Finally, the encapsulated ADP and membrane component (cholesterol) are eliminated mainly to the urine and feces, respectively. This successive disposition of H12-(ADP)-liposomes in thrombocytopenic conditions was the same as that in normal rats. However, the amount of urinary and fecal excretion of H12-(ADP)-liposomes and components derived from them after injection in thrombocytopenic rats were higher than that in normal rats with a decrease in blood retention and increment of hepatic distribution. As the thrombocytopenic rats used in this study reflect the clinical features of patients with thrombocytopenia induced by anticancer drugs, a similar phenomenon would be expected in clinical situations. The above findings provide useful information, such as dosing regimens, for the development of the H12-(ADP)-liposomes for use as a platelet substitute in future clinical applications.

## ACKNOWLEDGMENTS

We would like to thank Shunsuke Katsuno and Manami Arai at Waseda University for preparation of liposome samples. We also thank Mai Hashimoto at Kumamoto University for technical assistance in the present study. This work was supported in part by Health and Labor Sciences Research Grants (Research on Public Essential Drugs and Medical Devices) from the Ministry of Health, Labour and Welfare, Japan.

## REFERENCES


**1.** Blajchman MA. 2003. Substitutes and alternatives to platelet transfusions in thrombocytopenic patients. J Thromb Haemost 1(7):1637–1641.

**2.** Rybak ME, Renzulli LA. 1993. A liposome based platelet substitute, the plateletsome, with hemostatic efficacy. Biomater Artif Cells Immobilization Biotechnol 21(2):101–118.

**3.** Graham SS, Gonchoroff NJ, Miller JL. 2001. Infusible platelet membranes retain partial functionality of the platelet GPIb/IX/V receptor complex. Am J Clin Pathol 115(1):144–147.

**4.** Levi M, Friederich PW, Middleton S, de Groot PG, Wu YP, Harris R, Biemond BJ, Heijnen HF, Levin J, ten Cate JW. 1999. Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits. Nat Med 5(1):107–111.

**5.** Agam G, Livne AA. 1992. Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia. Eur J Clin Invest 22(2):105–112.

**6.** Casals E, Verdaguer A, Tonda R, Galan A, Escolar G, Estelrich J. 2003. Atomic force microscopy of liposomes bearing fibrinogen. Bioconjug Chem 14(3):593–600.

**7.** Coller BS, Springer KT, Beer JH, Mohandas N, Scudder LE, Norton KJ, West SM. 1992. Thromboerythrocytes. In vitro studies of a potential autologous, semi-artificial alternative to platelet transfusions. J Clin Invest 89(2):546–555.

**8.** Takeoka S, Teramura Y, Okamura Y, Handa M, Ikeda Y, Tsuchida E. 2001. Fibrinogen-conjugated albumin polymers and their interaction with platelets under flow conditions. Biomacromolecules 2(4):1192–1197.

**9.** Suzuki H, Okamura Y, Ikeda Y, Takeoka S, Handa M. 2011. Ultrastructural analysis of thrombin-induced interaction between human platelets and liposomes carrying fibrinogen gamma-chain dodecapeptide as a synthetic platelet substitute. Thromb Res 128(6):552–559.

**10.** Tokutomi K, Tagawa T, Korenaga M, Chiba M, Asai T, Watanabe N, Takeoka S, Handa M, Ikeda Y, Oku N. 2011. Decoration of fibrinogen gamma-chain peptide on adenosine diphosphate-encapsulated liposomes enhances binding of the liposomes to activated platelets. Int J Pharm 407(1–2):151–157.

**11.** Tokutomi K, Tagawa T, Korenaga M, Chiba M, Asai T, Watanabe N, Takeoka S, Handa M, Ikeda Y, Oku N. 2012. Ability of fibrinogen gamma-derived dodecapeptides with different sequences to bind to rat platelets. Int J Pharm 438(1–2):296–301.

**12.** Nishikawa K, Hagisawa K, Kinoshita M, Shono S, Katsuno S, Doi M, Yanagawa R, Suzuki H, Iwaya K, Saitoh D, Sakamoto T, Seki S, Takeoka S, Handa M. 2012. Fibrinogen gamma-chain peptide-coated, ADP-encapsulated liposomes rescue thrombocytopenic rabbits from non-compressible liver hemorrhage. J Thromb Haemost 10(10):2137–2148.

**13.** Okamura Y, Eto K, Maruyama H, Handa M, Ikeda Y, Takeoka S. 2010. Visualization of liposomes carrying fibrinogen gamma-chain dodecapeptide accumulated to sites of vascular injury using computed tomography. Nanomedicine 6(2):391–396.

**14.** Okamura Y, Katsuno S, Suzuki H, Maruyama H, Handa M, Ikeda Y, Takeoka S. 2010. Release abilities of adenosine diphosphate from phospholipid vesicles with different membrane properties and their hemostatic effects as a platelet substitute. J Control Release 148(3):373–379.

**15.** Okamura Y, Maekawa I, Teramura Y, Maruyama H, Handa M, Ikeda Y, Takeoka S. 2005. Hemostatic effects of phospholipid vesicles carrying fibrinogen gamma chain dodecapeptide in vitro and in vivo. Bioconjug Chem 16(6):1589–1596.

**16.** Okamura Y, Takeoka S, Eto K, Maekawa I, Fujie T, Maruyama H, Ikeda Y, Handa M. 2009. Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. J Thromb Haemost 7(3):470–477.

**17.** Taguchi K, Ujihira H, Ogaki S, Watanabe H, Fujiyama A, Doi M, Okamura Y, Takeoka S, Ikeda Y, Handa M, Otagiri M, Maruyama T. 2013. Pharmacokinetic study of the structural components of adenosine diphosphate-encapsulated liposomes coated with fibrinogen gamma-chain dodecapeptide as a synthetic platelet substitute. Drug Metab Dispos 41:1584–1591.

**18.** Walsh TJ, Yeldandi V, McEvoy M, Gonzalez C, Chanock S, Freifeld A, Seibel NI, Whitcomb PO, Jarosinski P, Boswell G, Bekersky I, Alak A, Buell D, Barret J, Wilson W. 1998. Safety, tolerance, and pharmacokinetics of a small unilamellar liposomal formulation of amphotericin B (AmBisome) in neutropenic patients. Antimicrob Agents Chemother 42(9):2391–2398.

**19.** Bekersky I, Fielding RM, Dressler DE, Kline S, Buell DN, Walsh TJ. 2001. Pharmacokinetics, excretion, and mass balance of $^{14}C$ after administration of $^{14}C$-cholesterol-labeled AmBisome to healthy volunteers. J Clin Pharmacol 41(9):963–971.

**20.** Taguchi K, Maruyama T, Iwao Y, Sakai H, Kobayashi K, Horinouchi H, Tsuchida E, Kai T, Otagiri M. 2009. Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. J Control Release 136(3):232–239.

**21.** Frank R, Hargreaves R. 2003. Clinical biomarkers in drug discovery and development. Nat Rev Drug Dis 2(7):566–580.

**22.** Yang C, Li YC, Kuter DJ. 1999. The physiological response of thrombopoietin (c-Mpl ligand) to thrombocytopenia in the rat. Br J Haematol 105(2):478–485.

**23.** Yamakawa N, Suemasu S, Watanabe H, Tahara K, Tanaka K, Okamoto Y, Ohtsuka M, Maruyama T, Mizushima T. 2013. Comparison of pharmacokinetics between loxoprofen and its derivative with lower ulcerogenic activity, fluoro-loxoprofen. Drug Metab Pharmacokinet 28(2):118–124.

**24.** Taguchi K, Miyasato M, Watanabe H, Sakai H, Tsuchida E, Horinouchi H, Kobayashi K, Maruyama T, Otagiri M. 2011. Alteration in the pharmacokinetics of hemoglobin-vesicles in a rat model of chronic liver cirrhosis is associated with Kupffer cell phagocyte activity. J Pharm Sci 100(2):775–783.

**25.** Kiwada H, Matsuo H, Harashima H. 1998. Identification of proteins mediating clearance of liposomes using a liver perfusion system. Adv Drug Deliv Rev 32(1–2):61–79.

**26.** Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, Horinouchi H, Kobayashi K, Tsuchida E, Kai T, Otagiri M. 2009. Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. Drug Metab Dispos 37(7):1456–1463.

**27.** Telgenhoff DJ, Aggarwal SK, Johnson BN. 1999. Histochemical and morphological identification of Kupffer cells activated by cisplatin. Anticancer Res 19(2A):1005–1010.

**28.** Coulson R. 1976. Metabolism and excretion of exogenous adenosine 3':5'-monophosphate and guanosine 3':5'-monophosphate. Studies in the isolated perfused rat kidney and in the intact rat. J Biol Chem 251(16):4958–4967.

**29.** Kuipers F, Spanjer HH, Havinga R, Scherphof GL, Vonk RJ. 1986. Lipoproteins and liposomes as in vivo cholesterol vehicles in the rat: Preferential use of cholesterol carried by small unilamellar liposomes for the formation of muricholic acids. Biochim Biophys Acta 876(3):559–566.

1521-009X/41/8/1584–1591$25.00
DRUG METABOLISM AND DISPOSITION
http://dx.doi.org/10.1124/dmd.112.050005
Drug Metab Dispos 41:1584–1591, August 2013


DRUG METABOLISM & DISPOSITION

# Pharmacokinetic Study of the Structural Components of Adenosine Diphosphate-Encapsulated Liposomes Coated with Fibrinogen γ-Chain Dodecapeptide as a Synthetic Platelet Substitute[S]

Kazuaki Taguchi, Hayato Ujihira, Shigeru Ogaki, Hiroshi Watanabe, Atsushi Fujiyama, Mami Doi, Yosuke Okamura, Shinji Takeoka, Yasuo Ikeda, Makoto Handa, Masaki Otagiri, and Toru Maruyama

*Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences (K.T., H.U., S.O., H.W., M.O., T.M.), and Center for Clinical Pharmaceutical Sciences (H.W., T.M.), Kumamoto University, Kumamoto, Japan; Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, Tokyo, Japan (A.F., M.D., S.T., Y.I); Institute of Innovative Science and Technology, Tokai University, Tokyo, Japan (Y.O.); Department of Transfusion Medicine & Cell Therapy, Keio University, Tokyo, Japan (M.H.); and Faculty of Pharmaceutical Sciences and DDS Research Institute, Sojo University, Kumamoto, Japan (M.O.)*

Received November 8, 2012; accepted June 4, 2013

## ABSTRACT

**Fibrinogen γ-chain (dodecapeptide HHLGGAKQAGDV, H12)–coated, ADP-encapsulated liposomes [H12-(ADP)-liposomes] were developed as a synthetic platelet alternative that specifically accumulates at bleeding sites as the result of interactions with activated platelets via glycoprotein IIb/IIIa and augments platelet aggregation by releasing ADP. The aim of this study is to characterize the pharmacokinetic properties of H12-(ADP)-liposomes and structural components in rats, and to predict the blood retention of H12-(ADP)-liposomes in humans. With use of H12-(ADP)-liposomes in which the encapsulated ADP and liposomal membrane cholesterol were radiolabeled with $^{14}C$ and $^{3}H$, respectively, it was found that the time courses for the plasma concentration curves of $^{14}C$ and $^{3}H$ radioactivity showed that the H12-(ADP)-liposomes remained intact in the blood circulation for up to 24 hours after injection, and were mainly distributed to the liver and spleen. However, the $^{14}C$ and $^{3}H$ radioactivity of H12-(ADP)-liposomes disappeared from organs within 7 days after injection. The encapsulated ADP was metabolized to allantoin, which is the final metabolite of ADP in rodents, and was mainly eliminated in the urine, whereas the cholesterol was mainly eliminated in feces. In addition, the half-life of the H12-(ADP)-liposomes in humans was predicted to be approximately 96 hours from pharmacokinetic data obtained for mice, rats, and rabbits using an allometric equation. These results suggest that the H12-(ADP)-liposome has potential with proper pharmacokinetic and acceptable biodegradable properties as a synthetic platelet substitute.**

## Introduction

As the numbers of patients with hematologic malignancies and solid tumors increase, platelet transfusion represents one of the most essential prophylactic or therapeutic treatments, because these disorders induce severe thrombocytopenia caused by the intensive chemotherapy, surgical procedures, and radiotherapy. However, platelet transfusion can introduce a variety of complications such as bacterial infection, allergic reaction, and acute lung injury. In addition, donated platelet for blood transfusions can only be stored for a period of 4 days in Japan and 5–7 days in the United States and Europe. This has become a serious concern in our aging society and a stable supply in an emergency situation such as disasters and pandemics needs to be on hand. To solve these problems, various platelet substitutes, which consist of materials derived from blood components, have been developed (Blajchman, 2003), such as solubilized platelet membrane protein conjugated liposomes (plateletsome) (Rybak and Renzulli, 1993), infusible platelet membranes (Graham et al., 2001), fibrinogen-coated albumin microcapsules (synthocyte) (Levi et al., 1999), red blood cells with bound fibrinogen (Agam and Livne, 1992), liposomes bearing fibrinogen (Casals et al., 2003), arginine-glycine-aspartic acid (RGD) peptide-bound red blood cells (thromboerythrocyte) (Coller et al., 1992), and fibrinogen-conjugated albumin polymers (Takeoka et al., 2001). However, these platelet substitutes have not yet been approved for clinical use.

ADP-encapsulated liposomes modified with a dodecapeptide (HHLGGAKQAGDV, H12) [H12-(ADP)-liposome] were developed as a new type of synthetic platelet alternative. The glycoprotein

This research was supported in part by the Japanese Ministry of Health, Labour, and Welfare [Health and Labor Sciences Research Grants on Research on Public Essential Drugs and Medical Devices to S.T., Y.I., M.H., and T.M.].
dx.doi.org/10.1124/dmd.112.050005.
[S]This article has supplemental material available at dmd.aspetjournals.org.

**ABBREVIATIONS:** CL, clearance; DHSG, 1,5-dihexadecyl-*N*-succinyl-L-glutamate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; GP, glycoprotein; H12, HHLGGAKQAGDV; H12-(ADP)-liposome, ADP-encapsulated liposomes modified with a dodecapeptide; HbV, hemoglobin vesicle; HPLC, high-performance liquid chromatography; ID, injected dose; MPS, mononuclear phagocyte system; PEG, polyethyleneglycol; PEG-DSPE, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[monomethoxypoly(ethyleneglycol)]; RGD, arginine-glycine-aspartic acid; $t_{1/2}$, half-life; $V_{dss}$, distribution volume.

(GP) IIb/IIIa, which is present on the platelet membranes, is converted from an inactive to an active form when platelets adhere to collagen that is exposed on sites of vascular injury (Takagi et al., 2002; Xiao et al., 2004), and platelet aggregation is mediated by fibrinogen by bridging adjacent platelets through GPIIb/IIIa in an activation-dependent manner in the circulation. Among several GPIIb/IIIa recognized sequence sites in fibrinogen such as the RGD-based sequences ($^{95}$RGDF$^{98}$ and $^{572}$RGDS$^{575}$ in the A$\alpha$ chains) and H12 ($^{400}$HHLGGAKQAGDV$^{411}$) in the carboxy-terminus of the $\gamma$-chain (Kloczewiak et al., 1982, 1984; Hawiger et al., 1989), H12 is a specific binding site of the ligand for activated GPIIb/IIIa (Lam et al., 1987; Andrieux et al., 1989), whereas RGD-related peptides are nonspecific with respect to a wide variety of integrins from various cell types (Phillips et al., 1991). In addition, when ADP is released from activated platelets, it functions as a potent platelet agonist. Thus, these modifications to H12-(ADP)-liposomes enable them to specifically interact with activated platelets, resulting in platelet aggregation. In fact, H12-liposomes with polyethyleneglycol (PEG)-surface modification specifically accumulate at the site of an injury in vivo and were determined to shorten bleeding time in a dose-dependent manner in a thrombocytopenic rat and a rabbit model (Okamura et al., 2005, 2009, 2010a,b; Nishikawa et al., 2012). Therefore, these findings prompted us to conclude that H12-(ADP)-liposomes have considerable potential for use as an alternative for actual platelets in clinical settings.

Before new drugs are approved for clinical use, they are required to undergo a wide variety of evaluations, including physicochemical tests, preclinical studies, and clinical trials. As described above, preclinical studies of H12-(ADP)-liposomes have resulted in pharmacological evidence to indicate that they can be used as a platelet substitute (Okamura et al., 2005, 2009, 2010a,b; Nishikawa et al., 2012). However, information concerning pharmacokinetic properties is lacking, especially the disposition and retention of each component in tissues after injection. Our strategy for the development of H12-(ADP)-liposomes is based on the fact that not only better pharmacological effects but also acceptable biodegradable properties (no accumulation or retention) need to be documented. In addition, preclinical pharmacokinetic studies in various mammalian species are essential, because the results of such studies can be extrapolated to humans, allowing appropriate dosing regimens to be estimated in the case of humans.

In the present study, we report on an evaluation of the pharmacokinetic properties of the H12-(ADP)-liposomes and components thereof, from the standpoint of stability in the blood circulation and the metabolism and excretion of each component. For this purpose, we prepared H12-(ADP)-liposomes that were $^{14}$C, $^{3}$H double radiolabeled, in which the encapsulated ADP and membrane component (cholesterol) were labeled with $^{14}$C and $^{3}$H, respectively. Furthermore, we predicted some important pharmacokinetic parameters, especially retention in the blood circulation, in humans, based on data obtained in pharmacokinetic studies in mice, rats, and rabbits.

## Materials and Methods

### Reagents

Cholesterol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) were purchased from Nippon Fine Chemical (Osaka, Japan), and 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[monomethoxypoly(ethyleneglycol)] (PEG-DSPE, 5.1 kDa) was from NOF (Tokyo, Japan). 1,5-Dihexadecyl-*N*-succinyl-L-glutamate (DHSG) and H12-PEG-Glu2C18, in which the fibrinogen $\gamma$-chain dodecapeptide (C-HHLGGAKQAGDV, Cys-H12) was conjugated to the end of the PEG-lipids, were synthesized as previous reported (Okamura et al., 2005). Allantoin, uric acid, hypoxanthine, xanthine, and ADP were obtained from Sigma-Aldrich (St. Louis, MO).

### Preparation of $^{14}$C, $^{3}$H Double-Labeled H12-(ADP)-Liposomes

First, $^{14}$C-labeled H12-(ADP)-liposomes were prepared under sterile conditions as previously reported, with minor modifications (Okamura et al., 2009). In brief, DPPC (1000 mg, 1.36 mmol), cholesterol (527 mg, 1.36 mmol), DHSG (189 mg, 272 $\mu$mol), PEG-DSPE (52 mg, 9.0 $\mu$mol), and H12-PEG-Glu2C18 (47 mg, 9.0 $\mu$mol) were dissolved in *t*-butyl alcohol and then freeze-dried. The resulting mixed lipids were hydrated with phosphate-buffered saline (pH 7.4) containing ADP (1 mM) and [8-$^{14}$C]ADP (1.85 MBq; Moravec Biochemicas Inc., La Brea, CA), and extruded through membrane filters (0.22 $\mu$m pore size, Durapore; Millipore, Tokyo, Japan). Liposomes were washed with phosphate-buffered saline by centrifugation (100,000*g*, 30 minutes, 4°C), and the remaining ADP was eliminated by sephadexG25. The diameter and $\zeta$ potential of the $^{14}$C-labeled H12-(ADP)-liposomes used in this study are regulated at 250 ± 50 nm and −10 ± 0.9 mV, respectively. The 5–10% of added ADP was encapsulated in the inner space of the vesicle.

The $^{3}$H labeling of $^{14}$C-labeled H12-(ADP)-liposomes, to prepare $^{14}$C and $^{3}$H double-labeled H12-(ADP)-liposomes, was carried out according to a previous report (Taguchi et al., 2009). The $^{14}$C-labeled H12-(ADP)-liposomes (1 ml) were mixed with [1,2-$^{3}$H(*N*)]cholesterol solution (10 $\mu$l), (PerkinElmer, Yokohama, Japan) and incubated for 12 hours at room temperature. $^{14}$C, $^{3}$H-labeled H12-(ADP)-liposomes were filtered through a sterile filter to remove aggregates (450 nm pore size). Before being used in pharmacokinetic experiments, all of the samples were mixed with unlabeled H12-(ADP)-liposomes. To utilize the same procedure using H12-(ADP)-liposomes and [1,2-$^{3}$H(*N*)]cholesterol, $^{3}$H-labeled H12-(ADP)-liposomes, which did not contain [8-$^{14}$C]ADP, were prepared for the pharmacokinetic studies in mice and rabbits.

### Animals

All animal experiments were undertaken in accordance with the guideline principle and procedure of Kumamoto University for the care and use of laboratory animals. Experiments were carried out with male ddY mice (28–30 g body weight; Japan SLC, Inc., Shizuoka, Japan), male Sprague-Dawley rats (180–210 g body weight; Kyudou Co., Kumamoto, Japan), and male New Zealand White rabbits (2.0–2.2 kg body weight; Biotek Co., Saga, Japan). All animals were maintained under conventional housing conditions, with food and water ad libitum in a temperature-controlled room with a 12-hour dark/light cycle.

### Pharmacokinetic Studies

**Administration and Collecting Blood and Organs in Rats.** Twenty-four Sprague-Dawley rats were anesthetized with diethyl ether and received a single injection of $^{14}$C, $^{3}$H-labeled H12-(ADP)-liposomes [10 mg lipids/kg ($n$ = 16), 20 mg lipids/kg ($n$ = 4), and 40 mg lipids/kg ($n$ = 4)]. In all rat groups, four rats were selected to undergo the plasma concentration test. Under ether anesthesia, approximately 200 $\mu$l blood samples in all administration groups were collected from tail vein at multiple time points after the injection of the $^{14}$C, $^{3}$H-labeled H12-(ADP)-liposomes (3, 10, and 30 minutes, and 1, 2, 3, 6, 12, 24, 48 and 168 hours) and the plasma was separated by centrifugation (3000*g*, 5 minutes). After collecting the last blood sample (168 hours), the rats were euthanized for excision of organs (kidney, liver, spleen, lung, and heart). Urine and feces were collected at fixed intervals in a metabolic cage. In addition, the four rats were sacrificed and organs were collected at 2, 6, and 24 hours after an injection of $^{14}$C, $^{3}$H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg.

**Administration and Collection of Blood and Organs in Mice and Rabbits.** Twenty-eight ddY mice received a single injection of $^{3}$H-labeled H12-(ADP)-liposomes (10 mg lipids/kg) in the tail vein under ether anesthesia. At each time after the injection of $^{3}$H-labeled H12-(ADP)-liposomes (3 and 30 minutes, and 1, 3, 6, 12, and 24 hours), four mice were anesthetized with ether and blood was collected from the inferior vena cava, and plasma was obtained by centrifugation (3000*g*, 5 minutes).

Four New Zealand White rabbits received a single injection of $^{3}$H-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg. The blood was collected from the auricular veins at each time after injection (3, 10, and 30 minutes, and

1, 2, 12, 24, 36, 48, and 72 hours), and plasma was obtained by centrifugation (3000*g*, 5 minutes).

**Measurement of $^{14}C$ and $^{3}H$ Radioactivity.** Plasma samples were solubilized in a mixture of Soluene-350 (PerkinElmer) and isopropyl alcohol (at a ratio of 1/1) for 24 h at 50°C. The organ samples were rinsed with saline, minced, and solubilized in Soluene-350 for 24 hours at 50°C. Urine and feces were also weighed and solubilized in Soluene-350. All samples were decolorized by treatment with a hydrogen peroxide solution after treatment of Soluene-350 or isopropyl alcohol. The $^{14}C$, $^{3}H$ radioactivity was determined by liquid scintillation counting (LSC-5121; Aloka, Tokyo, Japan) with Hionic-Fluor (PerkinElmer).

**Analysis of Metabolites of Encapsulated ADP.** ADP metabolites in urine were determined by high-performance liquid chromatography (HPLC), as described previously (George et al., 2006). A part of the urine obtained in the pharmacokinetic study in rats was used for this analysis, and aliquots of urine samples (2.5 ml) were mixed with 200 $\mu$l 10% sulfuric acid. Just before the analysis, the urine samples were centrifuged and filtered through a Dismic-25cs (0.2 $\mu$m pore size; ADVANTEC, Tokyo, Japan ) and diluted 10-fold with water after adjusting the pH to 7 with 0.01 N sodium hydroxide and 0.01 N sulfuric acid. A standard solution containing ADP, allantoin, uric acid, hypoxanthine, and xanthine was prepared as reported in a previous study (George et al., 2006). The HPLC system consisted of a Waters 2695 pump (Waters, Milford, MA), a Waters 2487 detector (Waters) operated at 220 nm. LC analyses were achieved with a 250 × 4 mm, 5 $\mu$m LiChrospher 100 RP-18 end-capped column (LiChroCART 250-4; Merck, Darmstadt, Germany). Furthermore, each ADP metabolite separated by HPLC was collected by a fraction collector (CHF121SA; ADVANTEC) and $^{14}C$ radioactivity was determined by liquid scintillation counting with Hionic-Fluor.

### Interspecies Scaling of Pharmacokinetic Parameters

Allometric relationships between various pharmacokinetic parameters (P) and body weight (W) were plotted on a log-log scale. Linear regression of the logarithmic values was calculated using the least-squares method using Eq. 1 (Boxenbaum, 1984).

$$P = \alpha \cdot W^{\beta} \quad (1)$$

P is the parameter of interest [distribution volume ($V_{dss}$) or clearance (CL)], W is the body weight (kg), and $\alpha$ and $\beta$ are the coefficient and exponent of the allometric equation, respectively. The average body weights of 0.034 kg (mouse), 0.242 kg (rat), 2.08 kg (rabbit), and 70 kg (human) were used for prediction of $V_{dss}$ and CL for human. After predicting of $V_{dss}$ and CL for humans (70 kg) using Eq. 1, the half-life for human was estimated.

### Data Analysis

A noncompartmental model was used for the pharmacokinetic analysis. Each parameter, including half-life ($t_{1/2}$, h), mean residence time (h), area under the concentration-time curve (h · % of dose/ml), CL (ml/h), and $V_{dss}$ (ml), was calculated using the moment analysis program available in Microsoft Excel (Microsoft Corporation, Redmond, WA) (Yamakawa et al., 2013). Data are shown as means ± S.D. for the indicated number of animals.

## Results

**Pharmacokinetics of H12-(ADP)-Liposome Components in Rats.** To investigate the pharmacokinetics of each component of the H12-(ADP)-liposomes, $^{14}C$, $^{3}H$-labeled H12-(ADP)-liposomes, in which the encapsulated ADP was labeled with $^{14}C$ and the membrane component (cholesterol) was labeled with $^{3}H$, were prepared (Fig. 1A). As shown in Fig. 1B and Table 1, the plasma concentration curves and pharmacokinetic parameters for $^{14}C$ radioactivity and $^{3}H$ radioactivity were similar. These data indicate that the structure of the H12-(ADP)-liposomes remained intact in the blood circulation for periods of up to 24 hours after injection in rats.

Moreover, we evaluated the tissue distribution of both the encapsulated ADP and membrane component (cholesterol) of the H12-(ADP)-liposomes. Figure 2 shows the tissue distribution in organs at 2, 6, and 24 hours after the administration of $^{14}C$, $^{3}H$-labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg to rats. Among these organs, the majority of both the $^{14}C$ and $^{3}H$ radioactivity of the H12-(ADP)-liposomes were distributed in the liver and spleen. However, both the $^{14}C$ and $^{3}H$ radioactivity of the H12-(ADP)-liposomes were eliminated from each organ, and the activity essentially disappeared within 7 days after injection (unpublished data). These data indicate that the H12-(ADP)-liposomes are mainly distributed to the liver and spleen, but the retention in these organs is negligible.

To identify the excretion pathway of the H12-(ADP)-liposomes, the levels of $^{14}C$ and $^{3}H$ in urine and feces were measured (Fig. 3, A and B). The $^{14}C$ was excreted mainly in the urine [80.4% ± 4.9% of the injected dose (ID) at 7 days after injection], but was low in feces (7.6% ± 2.7% of ID at 7 day after injection). On the other hand, the majority of the $^{3}H$ was excreted in the feces (74.2% ± 5.7% of ID at 7 days after injection), and excretion into the urine was essentially nil. In addition, as shown in Fig. 3C, it is well known that, in rodents, endogenous ADP is ultimately metabolized to allantoin and excreted. Thus, we qualitatively determined the fate of the encapsulated ADP of the H12-(ADP)-liposomes using an HPLC method. Figure 3D shows the separated peaks for ADP and its metabolites in the standard solution and in a urine sample 6 hours after the administration of the H12-(ADP)-liposomes to a rat. Furthermore, to exclude the effect of endogenous ADP and its metabolites, we measured the $^{14}C$ radioactivity of each peak that had been separated by HPLC. As a result, almost all of the $^{14}C$ radioactivity was detected in the peak corresponding to allantoin, which is the final metabolite of ADP in rodents, in the urine sample (Table 2).

These results indicate that more than 75% of each structural component of the H12-(ADP)-liposome is excreted from the body within 7 days after injection, and the encapsulated ADP and membrane component (cholesterol) derived from H12-(ADP)-liposomes were metabolized to final metabolites and excreted into the urine and feces, respectively.

**Dose-Dependence of H12-(ADP)-Liposomes Pharmacokinetics.** Figure 4 shows the time courses for the plasma concentration for the $^{14}C$, $^{3}H$-labeled H12-(ADP)-liposomes administered to rats at doses of 10, 20, and 40 mg lipids/kg. No significant difference was found in the plasma concentration curve or pharmacokinetic parameters among all groups (Fig. 4, A and B). In fact, a linear relationship between the administration dose and the area under the concentration-time curve was found, the values for which were calculated based on the lipids concentration (Fig. 4C). These data indicate that the disposition of the H12-(ADP)-liposomes is linear for a dose of 40 mg lipids/kg.

Moreover, the tissue distribution of both the encapsulated ADP and the membrane lipids component (cholesterol) of the $^{14}C$, $^{3}H$-labeled H12-(ADP)-liposomes was evaluated at 7 days after the injection of H12-(ADP)-liposomes at doses of 10, 20, 40 mg lipids/kg. The level of $^{14}C$ and $^{3}H$ radioactivity was nearly undetectable in the observed organs (kidney, liver, spleen, lung, and heart) (unpublished data). In addition, the radioactive $^{14}C$ was excreted mainly in the urine (80.4% ± 4.9%, 52.1% ± 3.6%, and 58.4% ± 7.1% of ID at 7 days after the injection at doses of 10, 20, and 40 mg lipids/kg, respectively), but was low in feces (7.6% ± 2.7%, 6.5% ± 2.9%, and 2.5% ± 1.9% of ID at 7 days after the injection at doses of 10, 20, and 40 mg lipids/kg, respectively). On the other hand, the majority of the radioactive $^{3}H$ was excreted in the feces (74.2% ± 5.7%, 98.9% ± 14.9%, and 70.6% ± 6.2% of ID at 7 days after the injection at doses of 10, 20, and 40 mg lipids/kg, respectively), and a small portion of the $^{3}H$ radioactivity was

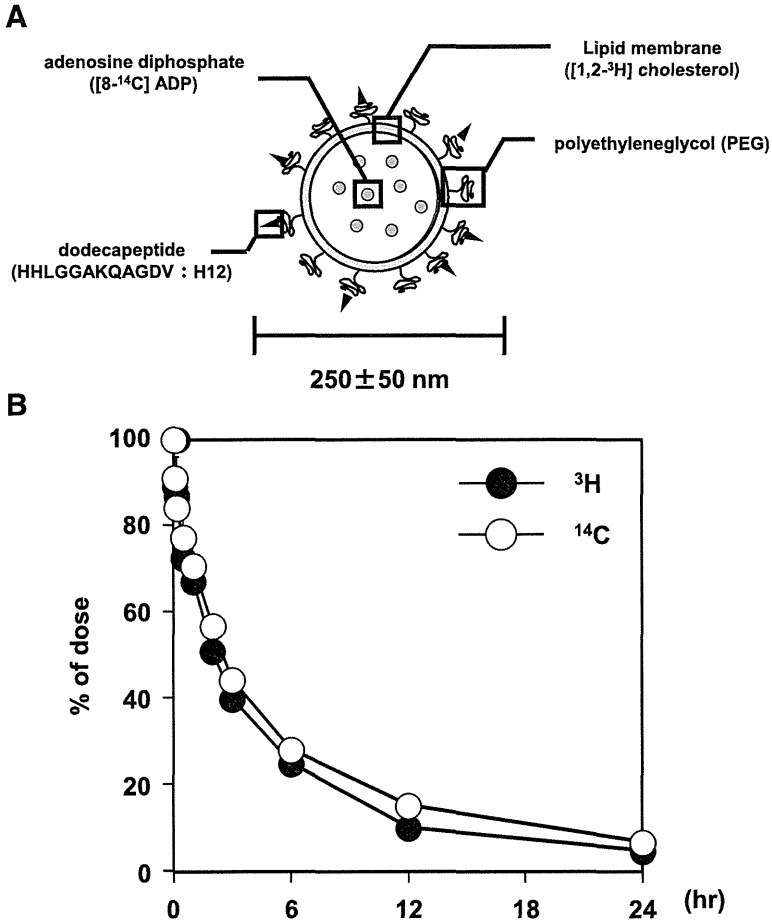


**Fig. 1.** (A) Structure and regiospecifically $^{3}H$- and $^{14}C$-radiolabeled H12-(ADP)-liposome. (B) Time course for the plasma concentration of $^{3}H$ and $^{14}C$ radiolabeled H12-(ADP)-liposome after intravenous injection at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. ($n$ = 4).

excreted into the urine. These data indicate that more than 75% of H12-(ADP)-liposomes are eliminated within 7 days after injection and retention in the body can be limited to detect at a dose of up to 40 mg lipids/kg.

**Pharmacokinetics of the H12-(ADP)-Liposomes in Mice and Rabbits.** To calculate the pharmacokinetic parameters of the H12-(ADP)-liposomes in mice and rabbits, the $^{3}H$-labeled H12-(ADP)-liposomes were administered to mice and rabbits at a dose of 10 mg lipids/kg. According to the pharmacokinetic parameters calculated from the plasma concentration curve, the CL and $V_{dss}$ of the $^{3}H$-labeled H12-(ADP)-liposomes in mice were 0.54 ± 0.12 ml/h and 3.81 ± 0.35 ml, respectively, whereas the values in the case of rabbits were 23.5 ± 2.8 ml/h and 827 ± 163 ml, respectively (Supplemental Table 1).

**Prediction of Pharmacokinetics of the H12-(ADP)-Liposomes in Humans.** To predict the pharmacokinetics in humans, we examined the allometric relationship between $V_{dss}$ and body weight (Fig. 5A) and CL and body weight (Fig. 5B) in mice, rats, and rabbits using the results summarized in Table 1 and Supplemental Table 1. As shown in Fig. 5, a good correlation in both relationships was observed. Furthermore, we calculated the half-life, based on extrapolation, of the H12-(ADP)-liposomes that were administered at a dose of 10 mg lipids/kg in humans to be approximately 96 hours.

TABLE 1

The pharmacokinetic parameters of inner ADP ([8-$^{14}C$]ADP) and outer lipid membranes ([1,2-$^{3}H(N)$]cholesterol) derived from $^{3}H$- and $^{14}C$-radiolabeled H12-(ADP)-liposomes after an intravenous injection at doses of 10, 20, and 40 mg lipids/kg to rats

Each value represents the mean ± S.D. ($n$ = 4).

| Parameter | 10 mg lipid/kg | | 20 mg lipid/kg | | 40 mg lipid/kg | |
|---|---|---|---|---|---|---|
| | $^{3}H$ | $^{14}C$ | $^{3}H$ | $^{14}C$ | $^{3}H$ | $^{14}C$ |
| $t_{1/2}$ (h) | 8.18 ± 0.77 | 8.21 ± 1.01 | 7.48 ± 0.56 | 7.63 ± 0.82 | 6.34 ± 0.53 | 7.94 ± 1.10 |
| MRT (h) | 10.2 ± 1.18 | 10.4 ± 1.46 | 9.20 ± 0.51 | 10.1 ± 1.00 | 7.65 ± 0.27 | 10.7 ± 0.97 |
| AUC (h · % of dose/ml) | 58.4 ± 6.45 | 54.2 ± 10.1 | 54.0 ± 1.97 | 67.8 ± 3.86 | 56.0 ± 1.94 | 84.4 ± 5.68 |
| CL (ml/h) | 1.73 ± 0.18 | 1.89 ± 0.32 | 1.85 ± 0.07 | 1.48 ± 0.08 | 1.79 ± 0.06 | 1.19 ± 0.07 |
| $V_{dss}$ (ml) | 17.7 ± 3.49 | 19.5 ± 3.61 | 17.0 ± 0.58 | 14.8 ± 0.64 | 13.7 ± 0.58 | 12.6 ± 0.98 |

AUC, area under the concentration-time curve; MRT, mean residence time.

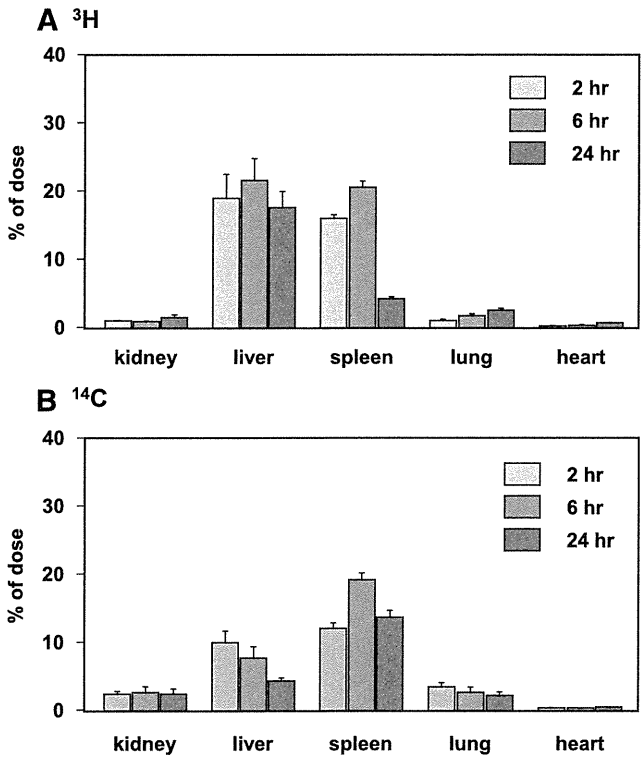


**Fig. 2.** The tissue distribution of $^{3}H$ (A) and $^{14}C$ radioactivity (B) at 2, 6, and 24 hours after an intravenous injection of $^{3}H$- and $^{14}C$-radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. ($n$ = 4).

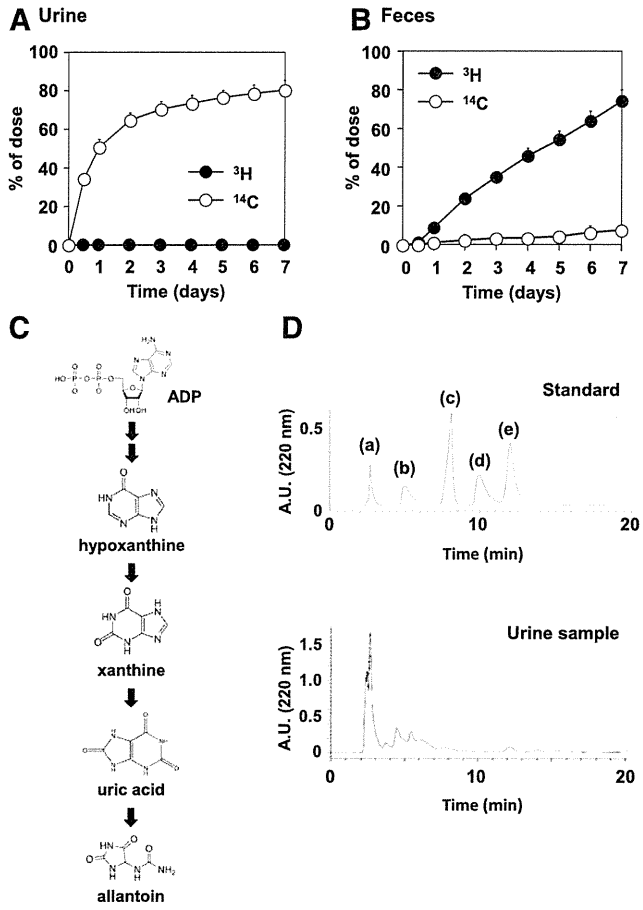


**Fig. 3.** Time course for radioactivity in urine (A) and feces (B) after the administration of $^{3}H$- and $^{14}C$-radiolabeled H12-(ADP)-liposome to rats. Each point represents the mean ± S.D. ($n$ = 4). (C) Scheme of metabolism pathway from ADP to allantoin in rodents. (D) Chromatogram of standard mixture and urine sample analyzed by HPLC. The standard peaks are (a) allantoin, (b) ADP, (c) uric acid, (d) hypoxanthine, and (e) xanthine. The urine sample was collected 6 hours after intravenous injection of $^{3}H$- and $^{14}C$-radiolabeled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats.

## Discussion

In the present study, the pharmacokinetic properties of H12-(ADP)-liposomes and structural components thereof, including the encapsulated ADP and membrane components (cholesterol), were characterized. The findings confirmed that the product has proper pharmacological functions and acceptable biodegradable properties (little retention). This leads to the conclusion that the H12-(ADP)-liposomes have the potential for use as a synthetic platelet substitute from the viewpoint of the pharmacokinetic properties in rodents.

We encapsulated ADP into H12 coated liposomes to strengthen the hemostatic ability of the H12 coated liposome as a platelet substitute, because this physiologically relevant platelet agonist is stored in dense granules and released upon cellular activation, and then functions to reinforce or maintain platelet aggregation through corresponding platelet nucleotide receptors P2Y1 and P2Y12. Thus, the stable encapsulation of ADP in liposomes permits them to function at sites of vascular injuries. The findings herein clearly show that, for up to 24 hours after injection in rats, the plasma concentration curves for $^{14}C$-, $^{3}H$-radiolabeled H12-(ADP)-liposome exhibited similar behaviors (Fig. 1), indicating that the H12-(ADP)-liposomes circulate in the bloodstream without any leakage of ADP. In addition, we also realized that the nonliposomal ADP was immediately eliminated from blood (unpublished data), because ADP released into blood was metabolized by leukocytes, erythrocytes, and endothelial cells (Marcus et al., 2003; Heptinstall et al., 2005). This means that ADP encapsulated in the vesicle has advantages that are not only specific delivery ADP to injury site but also improvement of the blood retention of ADP. Previous in vivo hemostatic studies of H12-(ADP)-liposomes using a rat model with busulphan-induced thrombocytopenia (platelet counts; $1.9 \pm 0.2 \times 10^{5}\ \mu l^{-1}$) clearly showed that the tail vein bleeding times of thrombocytopenic rats after an infusion of H12-(ADP)-liposomes (10 mg lipids/kg) were significantly reduced compared with that of controls [H12-liposome (10 mg lipids/kg) and (ADP)-liposome (10 mg lipids/kg)] (Okamura et al., 2009). Furthermore, the specific accumulation of H12-(iopamidol)-liposomes at the injury site at the rat tail vein and jugular vein were identified using an explore Locus CT system (Okamura et al., 2009, 2010a). These results indicate that the H12-(ADP)-liposomes circulate in the bloodstream in a stable form until reaching the site of a vascular injury, and successfully augments hematostatic effects.

Retention in the blood is also an important factor in the evaluation of the hematostatic effects of H12-(ADP)-liposomes, because if the systemic half-life of the H12-(ADP)-liposome is too short, it cannot effectively function as a platelet substitute. From the viewpoint of future clinical applications, an allometric prediction of human pharmacokinetics based on data obtained from animal studies—so-called "animal scale-up"—is important for the determination of optimal doses and intervals (Izumi et al., 1996). In fact, we successfully predicted the blood retention properties of hemoglobin vesicles (HbV), the liposomal characteristics of which have similar characteristics in terms of liposomal structure to H12-(ADP)-liposomes. This

TABLE 2

Time course for the percentage of total detected $^{14}C$ radioactivity of ADP and metabolites derived from $^{14}C$-ADP in urine after intravenous injection of $^{3}H$- and $^{14}C$-radiolabeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg to rats

Each value represents the mean ± S.D. ($n$ = 4).

| Time Course | Allantoin | ADP | Uric Acid | Hypoxanthine | Xanthine |
|---|---|---|---|---|---|
| 6 h | 89.7 ± 12.2 | 1.3 ± 1.2 | N.D. | N.D. | N.D. |
| 1 day | 78.7 ± 13.3 | 16.0 ± 15.8 | N.D. | N.D. | N.D. |
| 3 day | 71.8 ± 15.9 | N.D. | N.D. | N.D. | 11.2 ± 10.5 |
| 5 day | 75.0 ± 20.8 | N.D. | 3.9 ± 3.4 | N.D. | 6.7 ± 5.9 |

N.D., not determined.

was accomplished using an allometric equation that is generally applied in animal scale-up studies to extrapolate the half-life of pharmaceuticals in humans. In the present study, we showed that the predicted half-life of H12-(ADP)-liposomes in humans would be approximately 96 hours (Fig. 5) using the above approach. The results obtained for a single-dose pharmacokinetic study of recombinant factor VIIa (rFVIIa), which is widely used as a hemostatic agent in clinical settings, showed that its half-life was 2–3 hours in patients with hemophilia (Lindley et al., 1994). These results indicate that H12-(ADP)-liposomes would be expected to adequately function as a hemostatic agent in the treatment of massive bleeding in humans.

Since H12-(ADP)-liposomes were developed as a synthetic platelet substitute, it is necessary to characterize the biodegradable properties of these particles, such as the determination of their metabolism and excretion pathways. Liposomes are generally captured and degraded by mononuclear phagocyte system (MPS) in the liver and spleen, such as by Kupffer cells and splenic macrophages (Kiwada et al., 1998). As expected, more than 10% of initial dose of the H12-(ADP)-liposomes were distributed to the liver and spleen (Fig. 2), which is in good agreement with a previous in vivo study using HbV (Sakai et al., 2001, 2004). In addition, an in vitro finding also reported that the specific uptake and degradation of HbV were observed only in macrophage cells but not in parenchymal and endothelial cells in the liver (Taguchi et al., 2009). Furthermore, linear pharmacokinetics were found for the H12-(ADP)-liposomes within the dose of 40 mg lipids/kg (Fig. 4). These results strongly suggest that the majority of the H12-(ADP)-liposomes are also scavenged and degraded by the MPS, such as by Kupffer cells or splenic macrophages, and that this process was not saturated at a dose of 40 mg lipids/kg. However, it was observed the different amount of $^{3}H$ and $^{14}C$ distribution in liver and spleen (Fig. 2). This was similar to our previous finding using HbV that inner hemoglobin was rapidly eliminated from organs to urine and outer lipid component (cholesterol) was delayed to eliminate from organs to feces (Taguchi et al., 2009).Therefore, the different elimination pathway would be related to the retention in liver and spleen. Further study will be needed in this point.

The findings herein also showed that most of the ADP in H12-(ADP)-liposomes was mainly metabolized to allantoin and excreted into the urine within 7 days after the injection of the $^{14}C$, $^{3}H$-labeled H12-(ADP)-liposomes (Fig. 3). It is well known that uric acid is the final metabolite of purines, such as adenosine 3′,5′-phosphate, in mammals. On the other hand, the principal metabolite of exogenous cyclic nucleotides in the rat is allantoin, and not uric acid (Coulson, 1976). Furthermore, another study showed that, in rats, hepatic uricase

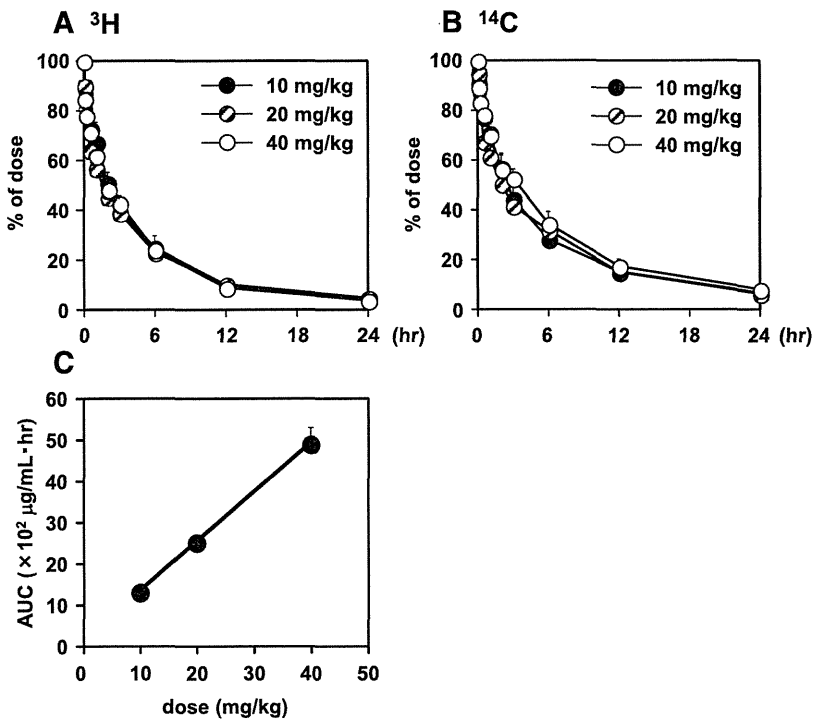


**Fig. 4.** Dose-dependent plasma concentration curve of (A) $^{3}H$- and (B) $^{14}C$-radiolabeled H12-(ADP)-liposome after intravenous injection at doses of 10, 20, and 40 mg lipids/kg to rats. Each point represents the mean ± S.D. ($n$ = 4). (C) Relationship between the dose of H12-(ADP)-liposome and the area under the blood concentration-time curve. The linear regression of logarithmic values was calculated using the least-squares method ($y$ = 98.33x+124.98, $r^2$ = 1)

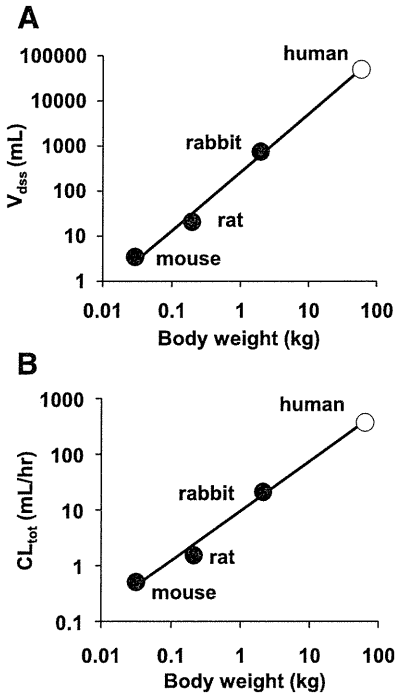


**Fig. 5.** Allometric relationships between body weight and distribution volume ($V_{dss}$) (A) and body weight and clearance (CL) (B). The linear regression of the logarithmic values was calculated using the least-squares method (A, $y = 257.71x^{1.2947}$, $r^2 = 0.965$; B, $y = 10.246x^{0.8928}$, $r^2 = 0.97$). The extrapolated human values based on a body weight of 70 kg (open circle) and the values from individual animals (gray circle) are also shown.

converts most of uric acid into allantoin, a form that allows it to be excreted in the urine more readily (Friedman and Byers, 1947). Taken together, these findings indicate that the ADP encapsulated by H12-(ADP)-liposome was completely metabolized and excreted into the urine even though ADP was encapsulated within liposome. However, $^{14}C$ radioactivity was not completely recovered until 7 days after $^{14}C$-labeled H12-(ADP)-liposome administration. Although we could not explain the reason why the recovery of $^{14}C$ radioactivity was less than 100% at higher doses, it was suggested that a part of encapsulated ADP was used in the body as endogenous ADP.

The [$^3$H]cholesterol in H12-(ADP)-liposomes was mainly excreted into feces within 7 days after the injection of $^3$H-labeled H12-(ADP)-liposomes. This result is in good agreement with the disposition of HbV, using HbV labeled with [$^3$H]cholesterol after an injection of HbV, which revealed that the majority of outer lipids component (cholesterol) was excreted via feces within 7 days (Taguchi et al., 2009). Kuipers et al. (1986) previously reported that cholesterol in vesicles reappear in the blood mainly as lipoprotein-cholesterol complexes after entrapment in Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein-cholesterol complex by hepatocytes. Therefore, a knowledge of whether the behavior of cholesterol as the lipid components of H12-(ADP)-liposome is the same as that of endogenous cholesterol after the metabolization of H12-(ADP)-liposome in the MPS would be highly desirable. On the other hand, we did not directly examine the disposition of the DPPC, DHSG, PEG-DSPE, and H12-PEG-Glu2C18 in H12-(ADP)-liposomes. Previous reports have shown that the phospholipids in liposomes are metabolized in the MPS and reused as cell membranes or are excreted into the bile (Dijkstra et al., 1985; Verkade et al., 1991). Therefore, it is also possible that phospholipids in H12-(ADP)-liposome are also metabolized and excreted in the same manner as the other liposome components, as mentioned above.

From the standpoint of biodegradable properties, it is also important to realize the possibility that H12-(ADP)-liposomes and components might accumulate in tissues, because it is well known that cholesterol is a risk factor for several diseases, including arteriosclerosis and hyperlipidemia. The findings reported herein indicate that both H12-(ADP)-liposomes and components derived from them disappeared from the bloodstream and organs within the 7 days after the injection of the H12-(ADP)-liposomes, indicating that H12-(ADP)-liposomes and components derived from them possess low accumulative properties. Therefore, H12-(ADP)-liposomes contain the appropriate components and have the potential for use as a synthetic platelet substitute, because they possess acceptable biodegradable properties.

Based on the present findings, we provide the first demonstration to show that the disposition of H12-(ADP)-liposomes and components derived from them occurs as follows. After being systemically administrated, the H12-(ADP)-liposomes are stable and circulate in an intact form in the circulation. As a result, some of the H12-(ADP)-liposomes would be specifically recruited at an injury site and would exert a pharmacological action, whereas the rest mainly are distributed to the liver and spleen, where they are degraded by the MPS. Finally, the encapsulated ADP and membrane components are eliminated mainly to the urine and feces, respectively, as final metabolites. In addition, our pharmacokinetic study, using different animal species, enabled us to predict that the half-life of H12-(ADP)-liposomes in humans is 96 hours. The above findings provide usable information for the development of the H12-(ADP)-liposomes for use as a platelet substitute.

**Acknowledgments**

The authors thank S. Katsuno and M. Arai at Waseda University for preparation of liposome samples.

**Authorship Contributions**

*Participated in research design*: Taguchi, Otagiri, Maruyama.
*Conducted experiments*: Taguchi, Ujihira, Ogaki, Fujiyama, Doi.
*Contributed new reagents or analytic tools*: Ikeda, Handa.
*Performed data analysis*: Taguchi, Ujihira, Watanabe.
*Wrote or contributed to the writing of the manuscript*: Taguchi, Okamura, Takeoka, Handa, Otagiri, Maruyama.

**References**

Agam G and Livne AA (1992) Erythrocytes with covalently bound fibrinogen as a cellular replacement for the treatment of thrombocytopenia. *Eur J Clin Invest* **22**:105–112.
Andrieux A, Hudry-Clergeon G, Ryckewaert JJ, Chapel A, Ginsberg MH, Plow EF, and Marguerie G (1989) Amino acid sequences in fibrinogen mediating its interaction with its platelet receptor, GPIIbIIIa. *J Biol Chem* **264**:9258–9265.
Blajchman MA (2003) Substitutes and alternatives to platelet transfusions in thrombocytopenic patients. *J Thromb Haemost* **1**:1637–1641.
Boxenbaum H (1984) Interspecies pharmacokinetic scaling and the evolutionary-comparative paradigm. *Drug Metab Rev* **15**:1071–1121.
Casals E, Verdaguer A, Tonda R, Galán A, Escolar G, and Estelrich J (2003) Atomic force microscopy of liposomes bearing fibrinogen. *Bioconjug Chem* **14**:593–600.
Coller BS, Springer KT, Beer JH, Mohandas N, Scudder LE, Norton KJ, and West SM (1992) Thromboerythrocytes. In vitro studies of a potential autologous, semi-artificial alternative to platelet transfusions. *J Clin Invest* **89**:546–555.
Coulson R (1976) Metabolism and excretion of exogenous adenosine 3′:5′-monophosphate and guanosine 3′:5′-monophosphate. Studies in the isolated perfused rat kidney and in the intact rat. *J Biol Chem* **251**:4958–4967.
Dijkstra J, van Galen M, Regts D, and Scherphof G (1985) Uptake and processing of liposomal phospholipids by Kupffer cells in vitro. *Eur J Biochem* **148**:391–397.
Friedman M and Byers SO (1947) Clearance of allantoin in the rat and dog as a measure of glomerular filtration rates. *Am J Physiol* **151**:192–197.
George SK, Dipu MT, Mehra UR, Singh P, Verma AK, and Ramgaokar JS (2006) Improved HPLC method for the simultaneous determination of allantoin, uric acid and creatinine in cattle urine. *J Chromatogr B Analyt Technol Biomed Life Sci* **832**:134–137.
Graham SS, Gonchoroff NJ, and Miller JL (2001) Infusible platelet membranes retain partial functionality of the platelet GPIb/IX/V receptor complex. *Am J Clin Pathol* **115**:144–147.

Hawiger J, Kloczewiak M, Bednarek MA, and Timmons S (1989) Platelet receptor recognition domains on the alpha chain of human fibrinogen: structure-function analysis. *Biochemistry* **28**: 2909–2914.

Heptinstall S, Johnson A, Glenn JR, and White AE (2005) Adenine nucleotide metabolism in human blood—important roles for leukocytes and erythrocytes. *J Thromb Haemost* **3**:2331–2339.

Izumi T, Enomoto S, Hosiyama K, Sasahara K, Shibukawa A, Nakagawa T, and Sugiyama Y (1996) Prediction of the human pharmacokinetics of troglitazone, a new and extensively metabolized antidiabetic agent, after oral administration, with an animal scale-up approach. *J Pharmacol Exp Ther* **277**:1630–1641.

Kiwada H, Matsuo H, and Harashima H (1998) Identification of proteins mediating clearance of liposomes using a liver perfusion system. *Adv Drug Deliv Rev* **32**:61–79.

Kloczewiak M, Timmons S, and Hawiger J (1982) Localization of a site interacting with human platelet receptor on carboxy-terminal segment of human fibrinogen gamma chain. *Biochem Biophys Res Commun* **107**:181–187.

Kloczewiak M, Timmons S, Lukas TJ, and Hawiger J (1984) Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the gamma chain. *Biochemistry* **23**:1767–1774.

Kuipers F, Spanjer HH, Havinga R, Scherphof GL, and Vonk RJ (1986) Lipoproteins and liposomes as in vivo cholesterol vehicles in the rat: preferential use of cholesterol carried by small unilamellar liposomes for the formation of muricholic acids. *Biochim Biophys Acta* **876**:559–566.

Lam SC, Plow EF, Smith MA, Andrieux A, Ryckwaert JJ, Marguerie G, and Ginsberg MH (1987) Evidence that arginyl-glycyl-aspartate peptides and fibrinogen gamma chain peptides share a common binding site on platelets. *J Biol Chem* **262**:947–950.

Levi M, Friederich PW, Middleton S, de Groot PG, Wu YP, Harris R, Biemond BJ, Heijnen HF, Levin J, and ten Cate JW (1999) Fibrinogen-coated albumin microcapsules reduce bleeding in severely thrombocytopenic rabbits. *Nat Med* **5**:107–111.

Lindley CM, Sawyer WT, Macik BG, Lusher J, Harrison JF, Baird-Cox K, Birch K, Glazer S, and Roberts HR (1994) Pharmacokinetics and pharmacodynamics of recombinant factor VIIa. *Clin Pharmacol Ther* **55**:638–648.

Marcus AJ, Broekman MJ, Drosopoulos JH, Islam N, Pinsky DJ, Sesti C, and Levi R (2003) Metabolic control of excessive extracellular nucleotide accumulation by CD39/ecto-nucleotidase-1: implications for ischemic vascular diseases. *J Pharmacol Exp Ther* **305**:9–16.

Nishikawa K, Hagisawa K, Kinoshita M, Shono S, Katsuno S, Doi M, Yanagawa R, Suzuki H, Iwaya K, and Saitoh D, et al. (2012) Fibrinogen γ-chain peptide-coated, ADP-encapsulated liposomes rescue thrombocytopenic rabbits from non-compressible liver hemorrhage. *J Thromb Haemost* **10**:2137–2148.

Okamura Y, Eto K, Maruyama H, Handa M, Ikeda Y, and Takeoka S (2010a) Visualization of liposomes carrying fibrinogen gamma-chain dodecapeptide accumulated to sites of vascular injury using computed tomography. *Nanomedicine* **6**:391–396.

Okamura Y, Katsuno S, Suzuki H, Maruyama H, Handa M, Ikeda Y, and Takeoka S (2010b) Release abilities of adenosine diphosphate from phospholipid vesicles with different membrane properties and their hemostatic effects as a platelet substitute. *J Control Release* **148**:373–379.

Okamura Y, Maekawa I, Teramura Y, Maruyama H, Handa M, Ikeda Y, and Takeoka S (2005) Hemostatic effects of phospholipid vesicles carrying fibrinogen gamma chain dodecapeptide in vitro and in vivo. *Bioconjug Chem* **16**:1589–1596.

Okamura Y, Takeoka S, Eto K, Maekawa I, Fujie T, Maruyama H, Ikeda Y, and Handa M (2009) Development of fibrinogen gamma-chain peptide-coated, adenosine diphosphate-encapsulated liposomes as a synthetic platelet substitute. *J Thromb Haemost* **7**:470–477.

Phillips DR, Charo IF, and Scarborough RM (1991) GPIIb-IIIa: the responsive integrin. *Cell* **65**: 359–362.

Rybak ME and Renzulli LA (1993) A liposome based platelet substitute, the plateletsome, with hemostatic efficacy. *Biomater Artif Cells Immobilization Biotechnol* **21**:101–118.

Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, and Tsuchida E (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* **159**:1079–1088.

Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suematsu M, Takaori M, Kobayashi K, and Tsuchida E (2004) Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* **311**:874–884.

Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, Horinouchi H, Kobayashi K, Tsuchida E, and Kai T, et al. (2009) Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. *Drug Metab Dispos* **37**:1456–1463.

Takagi J, Petre BM, Walz T, and Springer TA (2002) Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* **110**:599–611.

Takeoka S, Teramura Y, Okamura Y, Handa M, Ikeda Y, and Tsuchida E (2001) Fibrinogen-conjugated albumin polymers and their interaction with platelets under flow conditions. *Biomacromolecules* **2**:1192–1197.

Verkade HJ, Derksen JT, Gerding A, Scherphof GL, Vonk RJ, and Kuipers F (1991) Differential hepatic processing and biliary secretion of head-group and acyl chains of liposomal phosphatidylcholines. *Biochem J* **275**:139–144.

Xiao T, Takagi J, Coller BS, Wang JH, and Springer TA (2004) Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature* **432**:59–67.

Yamakawa N, Suemasu S, Watanabe H, Tahara K, Tanaka KI, Okamoto Y, Ohtsuka M, Maruyama T, and Mizushima T (2013) Comparison of pharmacokinetics between loxoprofen and its derivative with lower ulcerogenic activity, fluoro-loxoprofen. *Drug Metab Pharmacokinet* **28**:118–124.

**Address correspondence to:** Dr. Toru Maruyama, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: tomaru@gpo.kumamoto-u.ac.jp