

calculated by the following equation: volume = $\pi/6 \times$ width \times height \times length.

Statistical Analysis. The statistical significance of the data was evaluated by the Student's *t* test. $P \leq 0.05$ was considered significant.

RESULTS

Characterization of Microemulsions. Four kinds of ACM-loading microemulsions were formulated with M-ACM and 0.24 mol% of folate in F5/M-ACM, F2/M-ACM, and F0/M-ACM. All microemulsions were coated with PEG₂₀₀₀-DSPE to prolong the circulation time in the bloodstream. A schematic diagram shows that conjugated folate is located outside the coating PEG layer in F5/M-ACM, on the surface of the PEG-layer in F2/M-ACM, and inside the PEG-layer in F0/M-ACM (Fig. 2).

The average particle diameter of each microemulsion in water was ~120 nm and the ACM-loading efficiency was ~80% (data not shown). The average diameter and the ACM-loading efficiency of microemulsions did not change significantly for at least 1 month at 4°C in the dark. M-ACM was stable, showing the attenuated drug release, <50% release of drug for 48 hours in PBS (data not shown).

Expression of Folate Receptor Messenger RNA. We investigated the expression of FRs in the cell lines by reverse transcription-PCR. There were three FR isoforms, α , β , and γ , with distinctive patterns of tissue distribution. FR- α mRNA was strongly expressed in KB cells, but not HepG2 cells. FR- β and FR- γ mRNAs were not expressed in the cell lines (data not shown). This suggested that the cellular uptake of folate-linked microemulsion in KB cells was mediated via FR- α .

Cellular Association Determined by Flow Cytometry. We examined the selectivity of folate-linked microemulsions for delivery into KB cells using microemulsions labeled with DiI, a nonexchangeable fluorescent membrane probe, by flow cytometry.

As shown in Fig. 3, flow cytometry analysis represented a shift in the curve, indicating a clear increase in cellular association of F5/M and F2/M after 1-hour exposure. The mean fluorescence intensities of F5/M and F2/M had ~200- and 4-fold greater association than nonfolate microemulsion, respectively. In contrast, F0/M showed a similar curve to nonfolate microemulsion. These results correspond well with the idea that conjugating folate to a shorter PEG polymer reduces folate exposure by interference with the ability of liposome to interact with FR (8). Additionally, these

increased associations of F5/M and F2/M could be completely blocked by adding 2 mmol/L free folic acid to the medium (Fig. 4). From Fig. 2, one can assume that in F0/M-ACM, steric hindrances created by the emulsion surface or by long PEG chains attached to the emulsion interfere with FR recognition, whereas in F5/M-ACM and F2/M-ACM, there are no obstacles for such interaction. These findings are consistent with those previously reported on the receptor-dependent cellular uptake of folate-linked liposome (7, 19). These results suggest that microemulsions conjugated with folic acid outside the PEG layer can associate with the cells via FR.

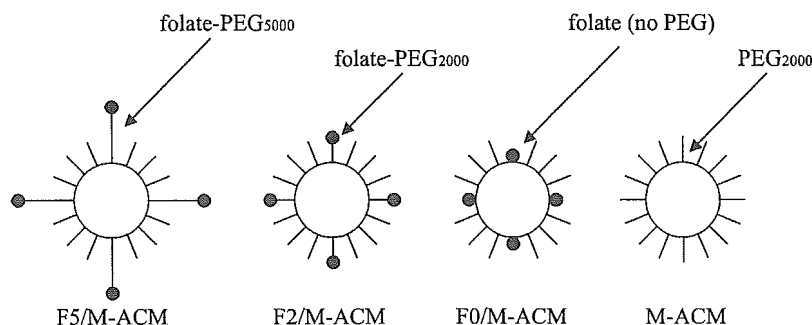
Cytotoxicity Studies. FR-targeted microemulsions were evaluated for *in vitro* cytotoxicity in FR- α (+) KB and FR- α (-) HepG2 cells by WST-8 assay. F5/M-ACM showed higher cytotoxicity than free ACM, M-ACM, and F2/M-ACM (Fig. 5A). M-ACM showed a slightly higher antitumor effect than free ACM in the FR- α (+) KB cells. In contrast, no therapeutic advantageous effect on cytotoxicity was observed with FR-targeted microemulsions in the HepG2 cell line (Fig. 5B), which is FR- α (-). Superior cytotoxicity of folate-linked microemulsion over M-ACM was observed in the FR- α (+) KB cells, but not in the FR- α (-) HepG2 cells.

ACM concentrations leading to 50% cell death (IC_{50}) by the microemulsions in KB cells were determined from concentration-dependent cell viability curves. Calculated IC_{50} values of F5/M-ACM, F2/M-ACM, and M-ACM were 0.8, 20, and 71 ng/mL, respectively, which were 150-, 6-, and 1.7-fold lower than free ACM (120 ng/mL). F5/M-ACM and F2/M-ACM were found to be 90- and 3.5-fold more cytotoxic than M-ACM, respectively. These results suggest that the microemulsions internalized into the cell release ACM and show an antitumor effect.

Serum Folate Concentration. The effect of dietary folic acid on serum concentration was investigated. In mice on a regular diet, serum folate remained at a relatively high level (203 nmol/L). Meanwhile, mice that were switched to a folate-deficient diet showed a significantly reduced serum folate concentration (70 nmol/L). Mice on a folate-deficient diet were actually able to maintain a plasma folate level around the human physiologic range (14-51 nmol/L; ref. 11).

Biodistribution of Microemulsions in Tumor-Bearing Mice. To reveal the tumor-targeting ability of folate-linked microemulsion-loading ACM, biodistribution of microemulsions was evaluated in mice bearing KB tumor xenografts. As shown in Fig. 6, the majority of microemulsions accumulated in the

Fig. 2 Schematic diagrams of the microemulsion and folate-linked microemulsions loading ACM. Folate-linked microemulsions loading ACM consisted of folate-linked lipids, PEG₂₀₀₀-DSPE, cholesterol, vitamin E, and ACM hydrochloride (0.24:6.7:48.3:43.3:1.5, molar ratio, F5/M-ACM, F2/M-ACM, and F0/M-ACM) were prepared by a modified ethanol injection method. For nonfolate microemulsions (M-ACM), folate-linked lipid was substituted with the same number of moles of PEG₂₀₀₀-DSPE. Closed circles, folate; straight lines, PEG chain.



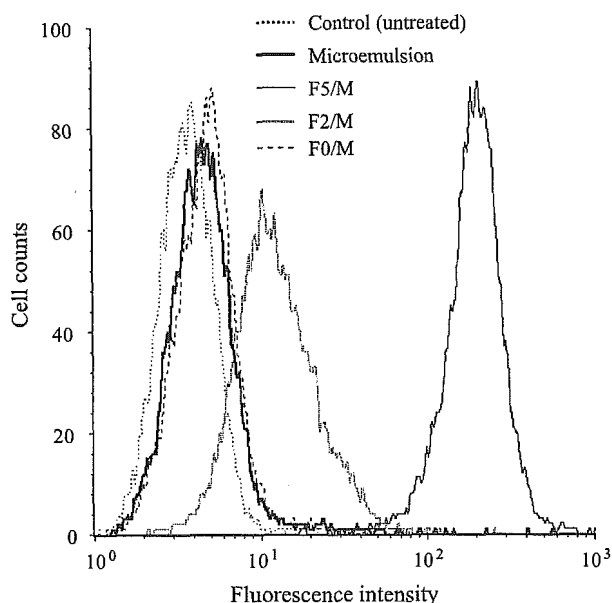


Fig. 3 Association of DiI-labeled F5/M, F2/M, F0/M, and microemulsion with KB cells. Cellular association was examined using DiI-labeled microemulsions (F5/M, F2/M, F0/M, and microemulsion) by flow cytometry. F5/M, F2/M, and F0/M represent folate microemulsion corresponding to F5/M-ACM, F2/M-ACM, F0/M-ACM without ACM, and microemulsion represents nonfolate microemulsion corresponding to M-ACM without ACM. Cells were incubated with microemulsions in serum-free medium at 37°C and analyzed by flow cytometry. Control indicates autofluorescence of untreated cells. Each analysis was generated by counting 10^4 cells.

spleen rather than in the tumor. The ACM concentrations of M-ACM in the plasma were ~1%, but that of F5/M-ACM and free ACM were not detected 24 hours after injection (data not shown). In each organ, ACM concentration was significantly higher in the order of F5/M-ACM, M-ACM, and free ACM, except in the lung. F5/M-ACM and M-ACM tended to accumulate in solid tumor regions 2.6- and 2.1-fold more than free ACM. These differences in tumor uptake reached statistical significance in F5/M-ACM to free ACM. There was no significant difference in tumor uptake between the folate- and

nonfolate-linked microemulsions. This finding suggests that folate-linked microemulsions loading ACM circulated longer than free ACM. High tumor accumulation of ACM from microemulsion may require 48 to 72 hours after i.v. injection (12).

Antitumor Effect. Biological antitumor activities of microemulsions were evaluated. Mice were given F5/M-ACM, F2/M-ACM, M-ACM, free ACM, and saline as a control every 3rd day, 5 times. As shown in Fig. 7, rapid tumor growth was observed in control mice. In contrast, tumor growth suppression was partially observed in all drug-treated mice. All microemulsions showed higher antitumor activity than free ACM, probably due to their long circulation and tumor targeting. The antitumor activity of F5/M-ACM was higher than that of F2/M-ACM. F5/M-ACM on day 21 and M-ACM on day 24 showed significantly higher tumor suppression than free ACM. Thus, F5/M-ACM seemed to be more effective than free ACM and nonfolate microemulsions in inhibiting tumor growth.

DISCUSSION

Recently, many reports have revealed the potential of FR-mediated drug delivery using folate-conjugated carriers. This strategy is based on the tumor cell specificity derived from the abundant expression of FR in a large percentage of human cancers. FR mediates the physiologic uptake of folate by endocytosis, and similarly actively internalizes conjugation with folate. To investigate FR targeting ability of folate-linked microemulsions, we used the similar experimental design and evaluation method with folate-linked liposomes that were reported (7–12). In this study, three kinds of folate-linked microemulsions, F5/M-ACM, F2/M-ACM, F0/M-ACM, and a nonfolate microemulsion, M-ACM, were prepared. These microemulsions were investigated and compared to reveal the targeting ability of PEG length in the folate-PEG linker chain both *in vitro* and *in vivo*. This study showed that folate modification with a sufficiently long PEG chain on emulsions is an effective way of targeting the emulsion to tumor cells.

This microemulsion was formed from a novel formulation. PEG-DSPE was used to stabilize the surface of droplets together with cholesterol (2). The folate-PEG modification microemulsion uses the same approach with liposomes reported by Lee and Low

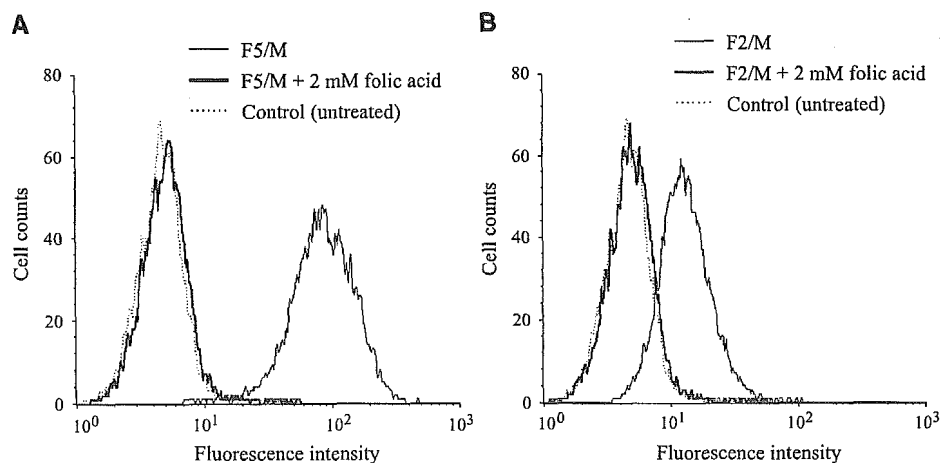
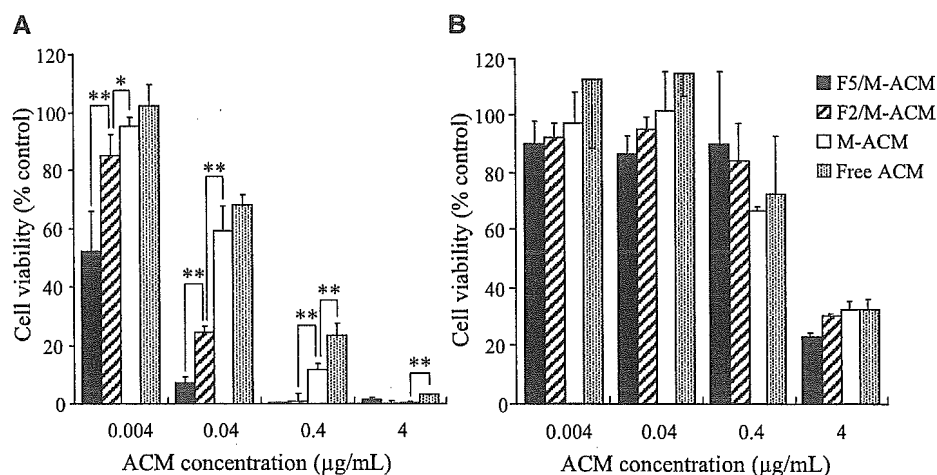


Fig. 4 Association of DiI-labeled F5/M (A) and DiI-labeled F2/M (B) with KB cells in the presence or absence of 2 mmol/L folic acid. Cells were incubated with DiI-labeled microemulsions in serum-free medium for 1 hour at 37°C. In free folate competition studies, 2 mmol/L folic acid was added to the culture medium. Control indicates autofluorescence of untreated cells in the absence of folic acid. Each analysis was generated by counting 10^4 cells. The curve shifts to the right, indicating an increase in the amount of DiI in the cells.

Fig. 5 Cytotoxicity of ACM delivered as F5/M-ACM, F2/M-ACM, M-ACM, or free ACM against KB cells (A) and HepG2 cells (B). Cells were incubated with drugs in serum-free medium for 1 hour, then in fresh medium without drugs for 72 hours at 37°C. Cell viability was assayed by the WST-8 method. Columns, mean; bars, SD ($n = 5-6$; * $P < 0.05$, ** $P < 0.01$).



(7, 20). Folate-targeted liposomes contained 0.1 to 0.5 mol% folate-PEG₂₀₀₀-DSPE or folate-PEG₃₃₅₀-DSPE, and ~4 mol% PEG₂₀₀₀-DSPE for PEG-coating of total liposome lipids (7–12). From this information, we decided to use 0.24 mol% folate-PEG₂₀₀₀-DSPE or folate-PEG₅₀₀₀-DSPE for targeting, and 6.7 mol% PEG₂₀₀₀-DSPE for PEG coating in the forming emulsion. For nonfolate-linked microemulsions, PEG₂₀₀₀-DSPE was used as M-ACM because increasing the PEG molecular mass from 1,900 to 5,000 had no effect for prolonged circulation (21). The folate-PEG-modified microemulsions were stable even after folate linked to microemulsion in the particle size and the amount of drug in the emulsion for at least 1 month at 4°C in the dark.

Folate-linked microemulsions were taken up by FR-mediated endocytosis. This was validated by the inhibition of association of folate-linked microemulsions in the presence of excess free folic acid and by selective FR-mediated cytotoxicity of folate-linked microemulsions loading ACM in KB and HepG2 cells.

When the results were compared among microemulsion formulations with cellular uptake by flow cytometry and cytotoxicity, the association of the folate-PEG₅₀₀₀-linked microemulsion and folate-PEG₂₀₀₀-linked microemulsion with the cells was 200- and 4-fold higher, whereas their cytotoxicity was 90- and 3.5-fold higher than those of nonfolate microemulsion, respectively. Other groups also reported both uptake enhancement and *in vitro* cytotoxicity enhancement in folate-linked liposomes. Lee and Low (7) reported that folate PEG₃₃₅₀/PEG₂₀₀₀-liposomes-entrapped doxorubicin showed ~1.6-fold higher uptake and 29-fold higher cytotoxicity than nonfolate liposomes in KB cells. Pan et al. (10) also reported that FR-β-targeted PEG₃₃₅₀/PEG₂₀₀₀-liposomes-entrapped doxorubicin showed a ~6.6-fold higher uptake by flow cytometry and a 25-fold higher cytotoxicity than the nonfolate one in KG-1 cells, respectively. It suggested that our folate-linked microemulsions were also highly FR-targeting carriers like liposomes.

PEG coating is mainly needed to extend systemic circulation *in vivo* to increase the possibility of FR targeting. The interference of the PEG coating with PEG₂₀₀₀ was more pronounced with folate-PEG₂₀₀₀-DSPE than with folate-PEG₅₀₀₀-DSPE. When the folate-PEG chain was PEG₅₀₀₀,

the association and cytotoxicity of emulsions were significantly increased compared with the nonfolate one as in the case of liposomes (8, 9). However, the following two findings in microemulsions showed differences from liposomes. First, nonfolate liposomes with a PEG-coating entrapped doxorubicin showed significantly lower cytotoxicity *in vitro* than the free drug because of the PEG coating (7, 9, 10). In contrast, the nonfolate microemulsion, M-ACM, showed a 1.7-fold higher cytotoxicity (IC₅₀) than free ACM. These findings are consistent with the fact that nonfolate emulsion loading paclitaxel showed a 1.2-fold higher level than the free drug in terms of cytotoxicity (15). Second, when the folate-PEG₂₀₀₀ chain length was the same in PEG₂₀₀₀-DSPE for the PEG coating, the association of liposomes with the cells was comparable with the nonfolate one from the labeled marker (8), but that of emulsions (F2/M) showed a 4-fold increase compared with nonfolate microemulsion (Fig. 3). The

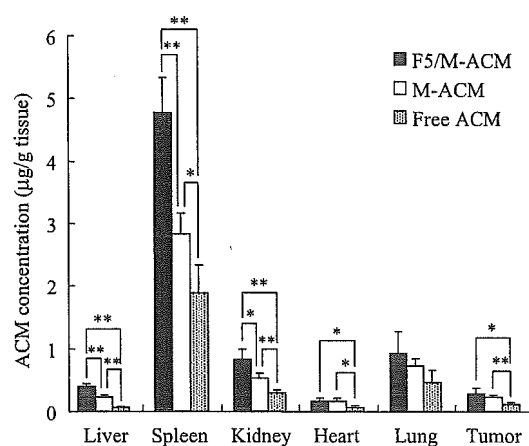


Fig. 6 Biodistribution of ACM 24 hours after i.v. injection of F5/M-ACM, M-ACM, and free ACM at a single dose of 5 mg ACM/kg into KB tumor-bearing BALB/c nude mice. Mice were inoculated s.c. with 2×10^6 cells into the right hind crus 7 days before the experiment. Mice were fed a folate-deficient diet from 1 week before tumor inoculation and for the duration of the study. Columns, mean; bars, SD ($n = 3$; * $P < 0.05$, ** $P < 0.01$).

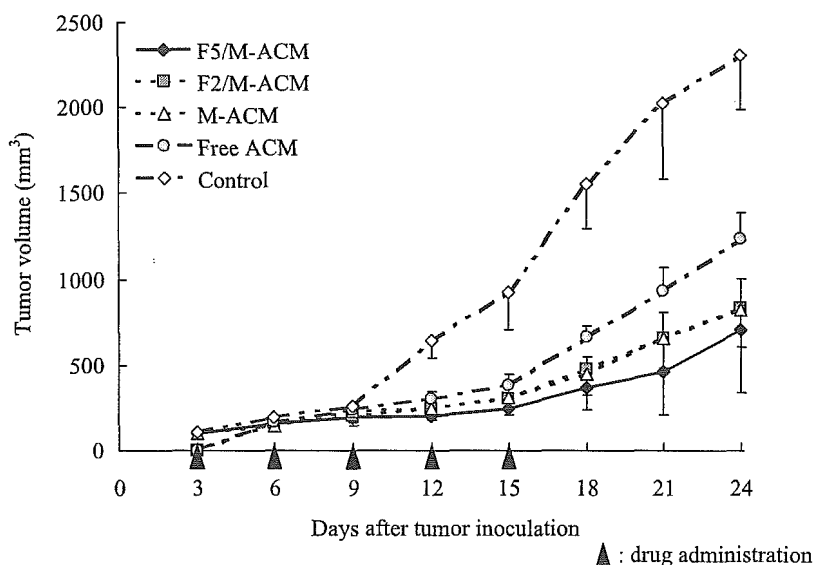


Fig. 7 *In vivo* antitumor effect by i.v. injection of F5/M-ACM, F2/M-ACM, and M-ACM at a dose of 5 mg ACM/kg in KB tumor-bearing BALB/c nude mice evaluated by solid tumor growth. Mice were inoculated s.c. with 1×10^6 cells into the right hind crus 3 days before the first administration of drugs. Mice were fed a folate-deficient diet from 1 week before tumor inoculation and for the duration of the study. Microemulsions were injected i.v. five times (day 3, 6, 9, 12, and 15) at a dose of 5 mg ACM/kg. The control was given the same volume of saline. Points, mean; bars, SD ($n = 3-6$).

mechanism for this difference between liposomes and microemulsions is unclear but this result implies that the interaction of PEG in carriers with the cells, and the release of a drug in them, might be different in spite of the similar PEG content (4 mol% in liposomes and 6.7 mol% in emulsions). PEG-DSPE in microemulsion was supposed to prolong circulation time *in vivo* as stabilizer and to form an injectable emulsion as emulsifier. Higher cytotoxicity of M-ACM than ACM suggests that M-ACM have some mechanism by which it achieves a high antitumor effect, such as circumvention of the efflux pump (9), because the release of ACM from M-ACM was attenuated (data not shown). Higher uptake of F2/M-ACM than M-ACM suggests that FR-targeted microemulsions are adsorbed and internalized quickly. Higher intracellular ACM fluorescence than M-ACM after 1-hour exposure of F2/M-ACM was also observed by confocal microscopy (data not shown).

The results from the biodistribution study revealed that the majority of i.v.-injected microemulsions accumulated in the liver and spleen rather than the tumor 24 hours after injection. The ACM concentrations of M-ACM in the plasma were ~1%, but F5/M-ACM and free ACM were not detected 24 hours after injection (data not shown). The level of ACM in the tumor of F5/M-ACM was shown to be similar to that of M-ACM and significantly higher than free ACM (Fig. 6). The higher accumulation of F5/M-ACM than that of M-ACM in the spleen might indicate that the folate residue counteracts the PEG-coating effect because the spleen constitutes a reticuloendothelial system. This result suggests the natural tendency of free ACM to accumulate in the spleen (22, 23).

In vivo experiments indicate that F5/M-ACM is also efficient *in vivo*. However, the advantages of F/M-ACM in terms of biodistribution and antitumor effect were relatively smaller than in *in vitro* experiments. Serum folate is not likely to have a significant inhibitory effect on the binding of folate-linked microemulsions to tumor cells expressing FR, because it was lower than the level required to produce significant

competition. For folate-linked microemulsions to bind FR, they require extravasation from the blood vessel in the tumor region, pass through the intercellular space, and then reach the FR on the cell surface. F5/M-ACM showed higher antitumor activity than M-ACM that showed significantly higher than free ACM on day 24. It was suggested that the circulation time was long enough for the present folate-linked microemulsion formulation to target tumors. In this way, one of the reasons for this tendency may be that FR uptake could be a rate-limiting factor due to the easy saturation of FR as reported in folate-targeted liposomes (24, 25).

In summary, this study suggests that folate-linked microemulsions offer a promising approach for tumor-targeted ACM delivery into FR-positive tumors. Extending the PEG chain length of the folate-PEG lipid from 0 to M_r 2,000 and 2,000 to M_r 5,000 dramatically improved the selective FR-mediated association, and cytotoxicity of folate-linked microemulsions loading ACM *in vitro*. Also, folate-linked microemulsions had a higher tumor-targeting efficacy and antitumor effect *in vivo*. Although these findings show that an improved therapeutic regimen of folate-linked microemulsions is needed, the novel folate-linked microemulsions will provide targeted tumor delivery of hydrophobic drugs *in vivo*.

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Application of Sterylglucoside-Containing Particles for Drug Delivery

Yoshie Maitani^{1,*}, Koji Nakamura² and Kumi Kawano¹

¹Institute of Medicinal Chemistry and ²Department of Pharmaceutics, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo, Japan 142-8501

Abstract: Recent advances in biotechnology have promoted biomolecular targeting of drugs, peptides and genes in the treatment and management of major diseases and infections. Therapeutic development of drugs and delivery systems may have various objectives: Systemic drugs require optimal delivery and uptake at target sites; peptide drugs require alternative routes of administration, such as nasal or intestinal absorption; gene medicines need to be delivered efficiently, safely and selectively to diseased areas. The propensity of ligand-modified liposomes to carry drugs and genes to desirable sites has been extensively examined and current reports show considerable progress in this field. Sterylglucoside (SG) is a novel absorption-enhancer of peptide drugs across nasal and intestinal mucosae. Physico-chemical properties and biodistribution of liposomes incorporating SG were studied and compared against the profiles of aglycon and sitosterol derivatives of SG. It was shown that SG particles aided colon drug delivery and increased bioavailability of peptide drugs after nasal and intestinal administration. In addition, they were able to enhance anticancer effects in liver cancer chemotherapy. Biological fate and interaction of SG with hepatocytes support the novel proposition of liver-targeting SG-liposomes.

Key Words: sterylglucoside; absorption enhancer; insulin; nasal absorption; nanoparticle; colon drug delivery; liver-targeting; anti-tumour drug.

INTRODUCTION

Recent advances in biotechnology have promoted molecular targeting of drugs, peptides and genes in the treatment and management of major diseases and infections. However, the administration routes and dosage forms of modern therapeutic drug delivery systems (DDS) have not evolved to complement and further such progress. Development of therapeutic DDS often aim for a variety of different goals. For example, peptide drugs are better suited to oral or nasal administration rather than systemic injection, and systemic drugs would benefit from optimal delivery and uptake at target sites.

Progress in biotechnology has led to the development of processes and the establishment of facilities for producing large quantities of peptide-based pharmaceuticals on an economical scale. This has made the therapeutic use of many peptide and protein pharmaceuticals feasible and practical. Since peptides and proteins are high molecular-weight macromolecules, they do not easily permeate into human intestinal mucosae. Oral or nasal administration exposes them to proteolytic enzymes in the gastrointestinal (GI) tract or nasal cavity, considerably reducing systemic bioavailability and efficacy. Penetration enhancers, such as bile salts and surfactants, may be co-administered to improve absorption. However, most types are harmful to intestinal and nasal epithelia. The search for safe and effective absorption enhancers is an important and on-going task.

Cancer is the highest cause of death all over the world. Conventional anticancer drugs are predominantly cytotoxic;

indiscriminate destruction of diseased and normal cells limits their therapeutic dose and routes of administration. The ability to target such drugs would be desirable since it would increase efficacy and decrease unwanted side-effects. Drug-targeting strategies may be classified as either "prodrug" or "carrier-mediated" systems. Pro-drugs are drug-related chemical precursors that become converted into their active forms at the site of action. Carrier-mediated systems involve either covalent or non-covalent association of drug molecules with a targeting moiety, which facilitates transport of drugs into target cells. Particulate systems, such as nanoparticles, nanospheres, emulsions, liposomes and mixed micelles, have been recently investigated as potential carrier systems for delivery and targeting of drugs to specific sites in the body. Modification of particles with specific ligands, such as antibodies, glycoconjugates, or peptides, enables active-targeting of tumour tissues.

The majority of absorption enhancers exhibit water-soluble properties, but a few can also be oil-soluble, such as fatty acids. Sterylglucoside (SG) is a soybean extract and comprises of four analogues (Fig. 1), primarily β -sitosterol β -D-glucoside (Sit-G), which exhibits slight solubility in water and in oil. Particulate SG exhibited the novel capability of promoting transport of peptide drugs through intestinal and nasal mucosae, as well as targeted delivery to hepatic tissues. The following topics will be discussed in detail:

1. Physicochemical properties of Sit-G nanoparticles (Sit-G NP) and liposomes containing SG (SG-liposomes), as compared with its aglycon.
2. Absorption enhancer: the efficacy of Sit-G NP and SG-liposomes in nasal and intestinal absorption of insulin and the mechanism of enhancer action.

*Address for correspondence to this author at the Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo, 142-8501 Japan; Tel/Fax: 81-3-5498-5048; E-mail: yoshie@hoshi.ac.jp

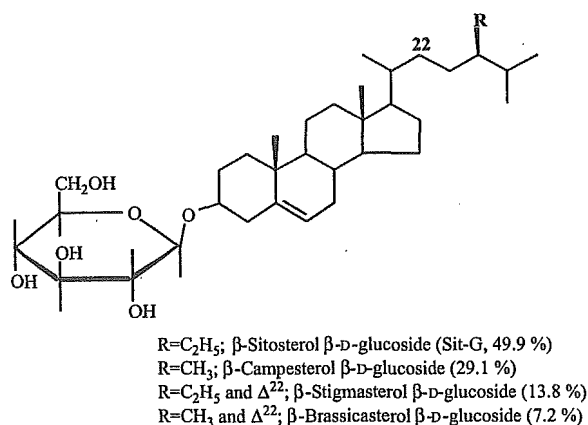


Fig. (1). Chemical structure of soybean-derived sterylglucoside (SG).

- Ligand for liver-targeting: the efficacy of SG-liposomes for liver-targeting *in vivo* and the mechanism pathway of SG activity in HepG2 cells.
- Application of SG-liposomes entrapping doxorubicin, pirarubicin and retinoic acid in cancer chemotherapy.

1. SOYBEAN-DERIVED STERYLGLUCOSIDE (SG)

Apart from functional variations at the C-24 position, phytosterols (plant sterols) exhibit structural similarities with the classical "animal sterol", cholesterol (Ch).

The type of SG used in this study was a mixture of glucoside analogues from soybean-derived sterol (SS): β-sitosterol (Sit, 49.9 %); campesterol (29.1 %); stigmasterol (13.8 %); and brassicasterol (7.2 %) (Fig. 1). Average molecular weight of SG is 570.5. Phytosterols are synthesized *de novo* in plants but their presence has been detected throughout the entire animal kingdom due to dietary consumption. As well as in animals, sterol concentrations in plants are high in plasma membranes and much lower in endoplasmic reticulum and mitochondria. In fact, 160.7 mg of SS is present per 100 g of edible soybean parts and total sterol in SS-derived oil is around 7.9 mg/g. Interestingly, SG itself is produced by transition of a glucose group from UDP-glucose to SS, and it makes up approximately 10% of all sterols present.

Favorable biological properties and a lack of toxicity have prompted much interest in using SG as an emulsifier and preservative in a wide variety of pharmaceutical applications and foodstuffs. For example, Bouic *et al.* [1-3] have been researching the effects of SS and SG on lymphocytes and their impact on diseases, including HIV, hepatitis, allergies, physical stress-induced immune suppression and inflammation. Interestingly, blood Ch can be reduced by drug treatment with SS in lipemic patients since it exhibits a higher affinity for biological membranes and competitively inhibits Ch uptake [4].

1.1. Sit-G Nanoparticles

Lipid particles have gained particular importance as a non-toxic and biodegradable carrier system for solubilizing

compounds that have low aqueous solubility, such as SG. Solubility of SG is 1.39 μg/mL in phosphate-buffered saline (PBS, pH7.4) at 37 °C. Nanoparticles and liposomes can be defined as colloid particles in the nanometer range of size and as phospholipid vesicles, respectively. A sample of Sit-G nanoparticles (Sit-G NP) incorporating oleic acid was prepared via modified ethanol-injection method. Dissociation of carboxylic groups on oleic acid created a negative charge on the surface of Sit-G NP. Particle size of Sit-G NP was dependent on the Sit-G amount in NP formulations. In the case of 0.5 % Sit-G NP, the size of NP was around 100 nm [5].

1.2. SG-Liposomes

Unlike polymeric and prodrug systems, nanoparticulate DDS offer the opportunity for improved absorption, as well as the capacity to transport relatively large quantities of drug. They can be effective carriers for site-specific delivery of drugs in oral and parenteral formulations. For example, liposomes incorporating sterylglucoside (SG-liposomes) can entrap labile drugs, e.g. insulin, protecting them from enzyme degradation.

When SG is incorporated into liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and Ch, the glucose residue of SG migrates to the outermost bilayer. This effect can be confirmed by the increase of turbidity of SG-liposomes after incubation with lectin [6,7], IR measurement [8] and quantifying glucose released from SG-liposomes after incubation with emulsin [8]. SG and its aglycon, SS could be the component as the liposomal membrane, and their effect on the liposomal membrane have the opposite ability: SS makes the liposomal membrane rigid compared with Ch [9], but SG makes high fluidity in the hydrophobic end of the liposomal membrane around 37°C, as revealed by fluorescence polarization analysis and ESR [10].

2. SG FOR ENHANCEMENT OF NASAL AND INTESTINAL ABSORPTION

2.1. Nasal Delivery

2.1.1. General Delivery Methods

Systemic delivery of peptide-based pharmaceuticals via nasal administration is a challenging task [11]. Drugs are poorly absorbed from nasal mucosa due to the available surface area for drug absorption in nasal mucosa being smaller than that in small intestinal or pulmonary epithelium. However, nasal drug delivery offers many advantages: The avoidance of the hepatic first-pass metabolism; rapid absorption due to relatively high tissue permeability; and ease of administration since the nasal cavity has a large and vascularized surface area. In practise, nasal delivery is considered when peroral drug delivery fails, such as in the case of peptides. Nasal administration is then often ranked as less effective when compared to pulmonary delivery, and better than transdermal delivery [12]. Currently, nasally-delivered therapeutics of low molecular weight peptides such as buserelin, desmopressin and calcitonin are on the market.

2.1.2. Barriers to Mucosal Absorption

Basic mechanisms of transepithelial transport of drugs include passive transport of small molecules, active transport

of ionic compounds, and endocytosis of macromolecules. Hydrophobic drugs will be predominantly absorbed by passive transport and directly permeate into lipid bilayers (transcellular transport). Cell membranes are relatively permeable to water and small hydrophobic molecules (paracellular transport) but their tight junction proteins restrict drug permeation by exerting a barrier-effect to macromolecules and most polar compounds. An inverse relationship was established between the extent of nasal absorption and molecular weight of absorbed hydrophilic compounds [13,14]. Another obstacle for drug permeation is the mucin layer at the surface of nasal mucosa. It acts as a diffusion barrier and washes out drugs and DDS material deposited on the nasal mucosa surface [15]. Another important factor is that proteolytic enzymes may destroy the therapeutic peptide before it permeates through the nasal mucosa.

2.1.3. Mechanisms of Action of Absorption Enhancers

In the last two decades, techniques for increasing drug bioavailability and improving absorption from mucosal membrane have centered on using absorption enhancers for avoiding extensive drug degradation and overcoming poor permeation. Methods to facilitate transport of molecules across epithelial cells can be categorized into two major groups: transcellular and paracellular transport. Most of the commonly used absorption enhancers influence both the transcellular and paracellular routes of absorption, albeit in different ratios. Several of these absorption enhancers are summarized in Table 1.

Table 1. Typical Absorption Enhancers

Enhancer type	Compounds
Paracellular	Chitosan EDTA Glycyrrhetic acid derivatives Saturated fatty acid (sodium caprate)
Transcellular	Bile salts Methylated β -cyclodextrin Saponins Unsaturated fatty acid (oleic acid)

Transcellular Transport

The mechanism of transcellular transport has yet to be elucidated. Fatty acid [16], monoglycerides [17], bile salts [14,18] and surfactants [19] have all been shown to increase epithelial membrane permeability by affecting the normal function of membrane proteins or lipids. A typical example of this type of enhancer is oleic acid. It increases the mucosal fluidity by partitioning into lipid bilayers and facilitates drug transport through mucosal membrane [20]. The most likely mechanism by which detergents enhance drug absorption is by solubilizing membrane phospholipids and proteins, thus increasing membrane permeability [19]. These actions may result in not only severe membrane damage, but also toxic side-effects due to absorption enhancers interfering with cell organelles.

Paracellular Transport

Paracellular transport is the transport of molecules around or between cells. Tight junctions or similar interconnections exist between cells. The intercellular tight junction is one of the major barriers to paracellular transport of macromolecules and polar compounds. Tight junction structure and permeability can be regulated by many potential physico-chemical factors, including the concentration of cAMP [21] and intracellular calcium concentrations [22]. Promotion of absorption by many Ca^{2+} chelators, such as EDTA, depends on their chelating activities [23]. Sodium caprate, a medium chain saturated fatty acid, induces an increase in intracellular calcium levels, resulting in the opening of tight junctions [24]. Glycyrrhizin can also trigger the opening of tight junctions and an increase in intracellular calcium levels [25].

Cyclodextrins can enhance the nasal absorption of peptide drugs, such as insulin. The mechanism of absorption enhancement of hydrophilic drugs by methylated β -cyclodextrins may be related to a temporary change in mucosal permeability (by extraction of membrane Ch) and opening of the tight junctions [26,27]. Poly-(acrylic acid) derivatives such as Carbomer 934[®] and chitosans have been extensively studied for their possible uses as absorption enhancers that cause the loosening of tight junctions [28,29].

2.2. Particulate Nasal and Oral Delivery System with SG

As already mentioned, the problems that make the oral route unsuitable for the systemic delivery of therapeutic peptides and proteins are the potential degradation by strong acids in the stomach and proteolytic enzymes in the intestinal tract, as well as pre-systemic elimination in the liver. Furthermore, it is known that macromolecular peptides and proteins have a very low permeability across GI mucosae. Thus, systemic bioavailability of peptide and protein pharmaceuticals by nasal and oral delivery is very low, generally less than 2%. Recently, great emphasis and study has been placed on particulate drug delivery as a novel type of DDS, that confers several advantages. First, the particle absorption process was found to be quite different from that of drug solutions transport would occur at the specialized mucosal membrane tissue, namely the mucosal associated lymphoid tissues (MALT) [30]. This transfer system may be utilized for vaccine and peptide delivery. Second, particles can migrate easily through mucous layers found on the surface of mucosae.

So far, there has only been a limited amount of information on particulate DDS incorporating absorption enhancers. The most successful examples of particulate delivery systems for nasal membranes were based on the absorption enhancers chitosan [31] and starch [32], which are bioadhesive polymers. Application of powders based on these materials increases their retention time on mucosal membranes. Liposomes have an inner aqueous phase surrounded by lipid bilayers. If peptides could be completely enveloped inside these liposomal, inner regions, they ought to be protected from attack by enzymes residing on the mucosal membrane.

2.2.1. Insulin

Systemic delivery of insulin, a protein hormone (amino acid 51, MW 5,806), by intranasal and oral administration has been studied extensively. Transmucosal permeability and mucosal absorption of insulin were found to be enhanced by co-administrating with absorption enhancers, such as bile salts (e.g., glyco- and deoxycholate), naturally occurring surfactants, or synthetic surfactants [12,15]. Numerous studies have shown that absorption enhancers can increase

the bioavailability of insulin (Table 2). However, bile salts caused mucosal irritation accompanied by lipid extraction [18]. Chitosan and cyclodextrin have also been shown to enhance insulin absorption, but they suppressed the ciliary beat of the mucosae [36,37].

Recent work showed that a hypoglycemia effect appeared just after nasal administration of powdered mixture of insulin, SG and SS. This decrease in glucose levels was greater than the 2.5% bioavailability after 10 IU/kg rabbit

Table 2. Comparison of Insulin Bioavailability After Co-Administration of Absorption Enhancer Following Nasal or Oral Delivery

Route	Enhancer	Dosage form (concentration or ratio) (size)	F (%)	Dose, Animal (F)	Ref
Nasal	Sodium deoxycholate	Spray (1 %)	20	0.9 IU/kg human	[33]
	Sodium glycocholate	Spray (4 %)	67.5	0.5 IU/kg human	
	Dimethyl β -cyclodextrin	Powder	12.9 \pm 4.4	4 IU/rabbit (0-3 h)	[26]
	Chitosan derivatives	Solution NP suspension (750 nm)	47.9 \pm 19.4	2.0 IU/kg rat (0-4 h)	[34]
			36.1 \pm 13.4		
	SS	SS suspension (1 %) Sit suspension (1 %)	5.3	10 IU/kg rabbit (0-6 h)	[42]
			5.5		
		DPPC/SS liposome (7/4) (100 nm)	5.1 \pm 0.5	7.0 IU/kg rabbit (0-21 h)	[41]
	SG	SG powder (physical mixture) SG powder (lyophilized powder)	2.6	10 IU/kg rabbit (0-6 h) 2 IU/kg rabbit (0-6 h)	[39]
			26.3 \pm 0.3		
SG suspension (1 %) Sit-G suspension (1 %)			6.7 \pm 1.4 11.3 \pm 1.6	10 IU/kg rabbit (0-6 h)	[42]
DPPC/SG liposomes (7/4) (100 nm) Freeze-dried DPPC/SG liposomes DPPC/SG liposome + insulin solution			13.3 \pm 5.7 24.2 \pm 5.3 15.7 \pm 3.2	7.0 IU/kg rabbit (0-8 h)	[41]
Oral	Chitosan	NP suspension (120 nm)	10.2 \pm 0.5	10 IU/kg rat	[35]
		NP suspension (350 nm)	14.9 \pm 1.3		
		NP suspension (1,000 nm)	7.3 \pm 0.8		
	Cholesterol	DPPC/Ch liposomes (7:2) (90 nm)	0.8 \pm 0.6	23.8 IU/kg rat (0-21 h)	[45]
		DPPC/Ch liposome (7:4) (110 nm)	21.9 \pm 5.8	15.5 IU/kg rat (0-21 h)	
	SS	DPPC/SS liposome (7:2) (150 nm)	24.1 \pm 4.9	28.3 IU/kg rat (0-21 h)	
		DPPC/SS liposome (7:4) (110 nm)	31.6 \pm 5.7	20.0 IU/kg rat (0-21 h)	
	SG	DPPC/SG liposome (7:2)	21.1 \pm 9.7	21.0 IU/kg rat (0-21 h)	
DPPC/SG liposome (7:4)		10.9 \pm 3.4			

F: pharmacological bioavailability (%), (F): pharmacological bioavailability from 0 h to final h, Sit: β -sitosterol, Sit-G: β -sitosterol β -D-glucoside, SS: soybean-derived sterol, SG: soybean-derived sterylglucoside, DPPC: dipalmitoylphosphatidylcholine, Ch: cholesterol, NP: nanoparticles.

achieved with a solution of insulin containing 1 w/v % sodium glycocholate [38]. The insulin powder dosage form with SG had been administered to the rabbit nasal cavity for five successive days [39]. Average bioavailability of insulin and the average pharmacological bioavailability at a dose of 2 IU/kg was approximately 25.0 and 61.6% after administration to 4 h, respectively (Fig. 2). Morphological observations revealed no severe damage to mucosal membranes [39,40]. These studies suggest that it is safe to use SG as an absorption enhancer for nasal delivery.

Nasal application of insulin-loaded liposomes triggered glucose reduction immediately after administration. Pharmacological bioavailability was greater and more prolonged in SG-liposomes than SS-liposomes; the hypoglycemia effect was persisted beyond 8 h after administration of SG-liposomes [41]. Insoluble powders and liposome dosage forms incorporating SG and SS improved bioavailability, predominantly by retarding drug elimination from the site of absorption and appeared to be suitable and effective for nasal systemic drug delivery when compared with liquid dosage forms. Comparing the insulin nasal absorption enhancement degree in main component (Sit-G) for SG and SG, Sit-G was stronger absorption ability than SG [42].

By the oral route, Gregoriadis *et al.* investigated the feasibility of systemic delivery of insulin using liposomes prepared from phosphatidylcholine and Ch [43]. A significant reduction in blood glucose levels was achieved in normal rats, with the maximum effect observed within 3 h. Patel *et al.* published the results of an extensive study on the delivery of insulin-entrapped liposomes to dogs, in the duodenal region, via a catheter [44]. Muramatsu *et al.* were able to produce a 31.6% reduction in blood glucose levels in normal rats via insulin-entrapped liposomes [45]. Oral application of insulin-loaded SS-liposomes brought glucose reduction from approximately 1 h to over 21 h after administration.

2.2.2. Erythropoietine

Erythropoietine (EPO) is a 30 kDa peptide. Oral administration of EPO-loaded liposomes incorporating SS caused a

dramatic increase in blood cell proliferation [46], but EPO-loaded liposomes incorporating SG did not. These data were consistent with insulin data above [45]. In general, liposome membrane stability may be an important determining factor on the effective GI absorption of peptides from oral particulate DDS.

2.2.3. FITC-Dextran

FITC-dextran with a varying range of molecular weights were used as a model drug to mimic the poor absorption of peptide drugs. Sit-G and Sit showed an increase in absorption enhancing effect after nasal administration of FITC-dextran 4,400 (FD-4) powder dosage form with them, and Sit-G showed higher bioavailability than Sit [47]. Comparing the degree of absorption enhancing effect of Sit-G and Sit with other absorption enhancers like cationic polymers, and bile salts, the enhancing degree of Sit-G and Sit was much weaker than cationic polymers, but in comparison of Sit and Sit-G with bile salts on bioavailability, there is no differences between them (Table 3).

Nanoparticles based on Sit-G and Sit were also tested to see how their particles would affect FD-4 absorption through nasal and intestinal mucosal membranes [47,49]. After nasal administration, the bioavailability of FD-4 with a particulate formulation was higher than powder or suspension preparations. Similarly, insulin nasal absorption was also facilitated by SG-liposomes, resulting in high hypoglycemic effect [41]. Preparations of Sit-G NP showed greater enhancement of absorption than Sit preparations [47]. These NP preparations included oleic acid in the formula, but NP based solely on oleic acid did not increase FD-4 absorption. By using NP formulations, it may be possible to ameliorate the hydrophobicity of Sit-G and Sit.

Sit-G NP enhanced FD-4 absorption predominantly in colon rather than rectum after co-administration of FD-4 with NP. The absorption-enhancing mechanism of NP will be described in the next section, but one hypothesis was that the distribution of β -D-glucosidase centred on colonic regions. Using this nature, dexamethazone β -D-glucoside has been developed as a prodrug for colon-specific delivery [50].

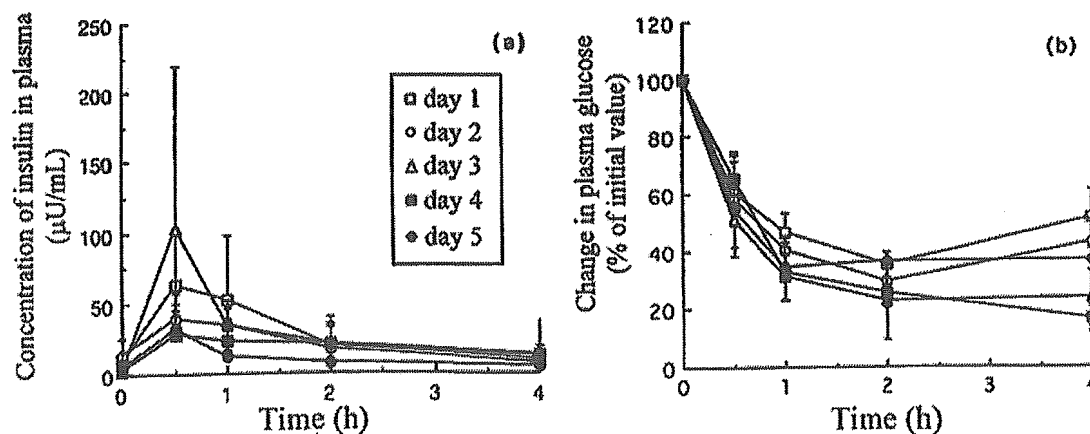


Fig. (2). Efficacy of porcine insulin powder with SG (insulin: SG= 2: 90) prepared by freeze-drying in a subchronic study.

Rabbits received drug for 5 successive days (2.0 IU/kg). Data represent blood insulin (a) and blood glucose (b) on day 1,2,3,4, or 5 (mean \pm S.D.; n=3) [39].

Table 3. Influence of Absorption Enhancer on FD-4 Absorption

Route	Enhancer	Dosage form (size)	F (%)	Dose, Animal (F)	Ref	
Nasal	EDTA	0.5 % solution	5.7	33.0 mg/kg rat (0-9 h)	[48]	
	Glycocholate	0.5 % solution	8.8			
	Chitosan	0.5 % solution	24.4			
	Sodium caprate	1.0 % solution	8.2 ± 2.9	1 mg/kg rabbit (0-3 h)	[47] [49]	
	Sit ^{a)}	powder (< 75 μm) ^{b)}	6.9 ± 0.4] *] *
		0.05 % suspension NP suspension (100 nm)	5.8 ± 0.9* 9.2 ± 0.8**			
Sit-G ^{a)}	powder (< 75 μm) ^{b)}	8.3 ± 1.0				
	0.05 % suspension NP suspension (100 nm)	8.9 ± 0.6** 11.7 ± 0.7**				
Ileum ^{c)}	Sit	NP suspension	6.3 ± 2.0	1 mg/kg rat (0-3 h)	[5]	
	Sit-G	0.05% suspension (< 75 μm) NP suspension (100 nm)	6.4 ± 1.0 5.4 ± 2.4			
Colon ^{d)}		Sit	NP suspension (100 nm)			3.1 ± 0.7
	Sit-G	0.05% suspension (< 75 μm) NP suspension (100 nm)	3.4 ± 0.2 15.4 ± 1.8 **			
Rectum ^{e)}		Sit	NP suspension (100 nm)			2.7 ± 0.9
	Sit-G	0.05% suspension (< 75 μm) NP suspension (100 nm)	3.1 ± 0.7 5.8 ± 1.2 *			

F: bioavailability (%), (F): bioavailability from 0 h to final h, NP: nanoparticles.

FD-4 solution in PBS was applied as control: ^{a)} F = 2.6 ± 0.4 (%), ^{b)} F = 5.6 ± 1.5 (%), ^{c)} F = 2.6 ± 0.6 (%), ^{d)} F = 2.2 ± 0.1 (%), ^{e)} FD-4 powder as control: F = 4.1 ± 1.0 (%), **; p<0.01, *; p<0.05, compared with control.

2.3. Absorption-Enhancing Mechanism of SG

Various factors have been studied in an attempt to elucidate the mechanisms involved with absorption enhancement. Changes in transepithelial electrical resistance (TEER) after application of absorption enhancers have been linked to a relaxation of tight junctions along the paracellular route. Assessment of TEER was used to correlate intracellular calcium flux with regulation of tight junctions in these cells [22]. Typical paracellular-type enhancers, like sodium caprate, induce an increase in intracellular calcium levels irrespective of extracellular calcium ion levels, causing tight junctions to open [51]. Application of Sit-G did not change TEER values at all after 2 h timepoint. Post-administration, intracellular calcium levels were elevated only in the presence of calcium ions in the extracellular space [52]. These results suggest that the increase in intracellular calcium ion levels may be a secondary effect of membrane disruption caused by Sit-G interactions. From this perspective, SG would act as an absorption enhancer in the transcellular category.

Nevertheless, Sit-G NP caused a significant decrease in mucosal membrane resistance (TEER) immediately after application of Sit-G NP onto either nasal or intestinal mucosal membranes [5,49]. Specific inhibition of sodium-dependent glucose transporters (SGLT) suppressed this reduction (Fig. 3), but did not affect FD-4 permeation [5]. Surprisingly, treatment of mucosal membranes with

endocytosis inhibitors made the absorption enhancing effect of Sit-G NP completely disappear. This effect by Sit-G NP may be due to an endocytotic process and a glucose moiety resides on Sit-G NP because Sit NP did not exhibit such behaviour.

Glucose transporters are widely distributed and can be separated into two categories: SGLT and GLUT, a non-dependent glucose transporter. Type SGLT exhibits inherent properties and can activate a SGLT-related decrease in TEER, since sodium ions become co-transported into cells when SGLT recognize glucose [53]. Recognition of glucose residues in SG by SGLT may be due to propensity of NP and liposomes to migrate into the mucus or microvillae at the surface of cell membranes.

Nasal absorption of insulin-loaded SG-liposomes caused a greater hypoglycemic effect than similar SS-liposomes [41]. Initially, it was thought that this difference might be due to liposomal release of insulin as a result of membrane destabilisation. Membranes within SG-liposomes are less rigid, making insulin easily released at partitioning junctures within SG-liposomes. This might raise the typical insulin concentration gradient higher since diffusion of insulin solution would have been restricted. On the other hand, since the membranes of SS-liposomes were stable, they would not release their drug load. When attempting oral delivery of peptides using liposomes, the rigidity of liposomal membranes may play an important factor since the relative

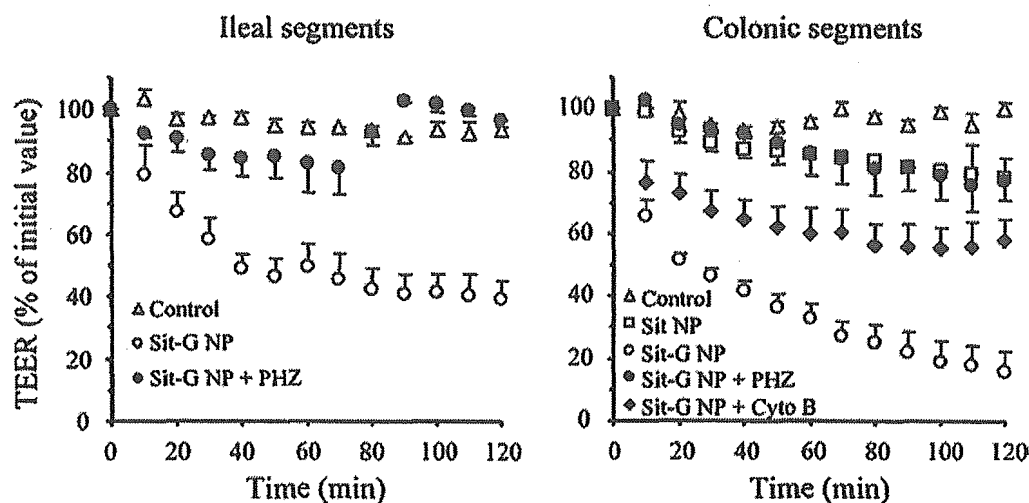


Fig. (3). Comparison of membrane resistance (TEER) after application of 0.05% Sit-G NP without or with SGLT inhibitor (PHZ) or endocytosis inhibitor (Cyto B) to the mucosal side in excised ileal and colonic mucosae in rats.

Each value was represented as the mean \pm SD. (n=4). Control: Ringer's solution.

Phloridzin (PHZ) was treated on the mucosal side before adding Sit-G NP.

Cytochalasin B (Cyto B) at a concentration of 20 μ M was added to the mucosal side.

Sit-G NP enhanced FITC-dextran 4,400 absorption in colonic segments, but not in ileal segment. [5].

distance from the administration site to the absorption site must be taken into consideration.

A second explanation for absorption enhancement of insulin by SG-liposomes is that an interaction occurs between Sit-G NP and certain bindings located at the surface of mucosal membranes. Indeed, SG-liposomes themselves facilitated absorption, as demonstrated by the faster onset of hypoglycemia from a mixture of empty SG-liposomes (insulin free liposomes) plus insulin solution compared to a preparation of insulin-loaded SG-liposomes [41]. Thus, a combination of these factors, such as the rate of insulin release and the absorption enhancement of SG-liposomes, may result in a potentiated hypoglycemic effect.

Normally, insulin at a neutral pH would form a hexamer state, but the addition of bile salts may be capable of dissociating insulin oligomers to monomers [54]. However, the trace in a circular dichroism spectra of insulin did not show any change in the conformation of insulin after an application of SG [42]. Co-administration of Sit and Sit-G with other drugs, e.g. verapamil [52] and FD-4 [47], showed the same behaviour with insulin, where their joint application resulted in an increase in insulin absorption that was substantially higher in SG than SS. Therefore, the absorption enhancement characteristics of SG and SS were not specific for just insulin and their mechanisms may be due to an interaction with mucosal membrane structures.

3. SG-LIGAND FOR LIVER-TARGETING

3.1. Liposomes for Liver Targeting

Drug targeting can be applied for therapeutic and for diagnostic purposes. The primary aim of drug targeting for

therapeutic use is to manipulate the whole-body distribution of drugs. That is, to prevent distribution to non-target cells and concomitantly increase the drug concentration in target cells. Low-molecular-weight compounds have high vascular permeability, resulting in homogenous distribution in all tissues after intravenous injection. On the other hand, polymers and particles cannot efficiently cross the endothelial cell barrier in most normal tissues because the diameters of capillary pores in normal tissues are generally less than 80 nm. Therefore, after intravenous injection of polymers and particles, they distribute to specific tissue types, depending on the vascular structure. The liver and spleen possess small pores called fenestrae, which are sufficiently large to allow particles to escape the circulation. In these regions, particles smaller than 100 nm can leave the general circulation and be taken up by hepatocytes. However, capillaries in these regions are lined with active macrophages, called Kupffer cells, which remove most circulating particles. The majority of injected colloidal particles will end up in Kupffer cells rather than hepatocytes. Uptake and clearance by reticuloendothelial system (RES) is a major barrier for colloidal particles when used for intravenous targeting applications.

To defeat RES uptake and increase circulation time in blood, one of the few promising approaches is the use of long-chain hydrophilic polymers (polyethylene glycol, PEG) to form a heavily hydrated layer around the particle. The mechanism for escaping the RES is not clear, but is thought to be related to preventing adsorption of serum recognition factors, or opsonins, which mediate the uptake process.

An advantage of using particle carriers, such as liposomes, is that drugs can easily become encapsulated, either dissolved in the aqueous phase or in the lipid phase, without

the requirement of a covalent linkage between drug and carrier. Liposomal surfaces may be easily modified with specific targeted ligands, such as monoclonal antibodies, sugar residues or proteins. A summary of ligands recognized by receptors present on the sinusoidal membrane of liver is displayed in Table 4. The asialoglycoprotein receptor (ASGP-R), only found on hepatocytes [55,56], has been widely used to selectively deliver compounds to hepatocytes by exploiting the presence of galactosylated proteins. Galactose-terminated compounds, such as lactosylceramide (LC) [57,58], asialofetuin (AF) [59,60] or synthetic glycolipids [61] have been used to modify liposomes for hepatocyte-specific drug delivery. Mannose residues were used to target Kupffer or non-parenchymal cells [62-64], and can be useful for delivering immunomodulators such as muramyl peptides [65] and gamma-interferon [66]. Negatively-charged (succinylated or aconitylated) albumins have been used to target scavenger receptors [67].

Table 4. Ligands Recognized by Endocytosis Receptors on the Cell Surface of the Hepatic Cells and Used for Drug Targeting Preparations

	Ligands for endocytosis receptors
Hepatocyte	Asialoglycoprotein (ASGP) Insulin Epidermal growth factor (EGF) Low density lipoprotein (LDL) High density lipoprotein (HDL) IgA Transferrin
Kupffer cell	Mannose Acetylated LDL Fc (immunocomplex) Fibronectin α_2 -Macroglobulin
Endothelial cell	Mannose Polyanion (scavenger) Fc

3.2. *In Vivo* Efficacy of SG-Liposomes for Targeting Hepatocytes

In recent years, liposomes modified with SG (SG-liposomes) have been reported as effective, liver-targeting carriers [6,68]. Compared with bare liposomes (DPPC alone), SG-liposomes (DPPC/SG=7/2) entrapped with fluorescent dye calcein showed an increased accumulation in the liver of mice after intravenous injection (Fig. 4). The level in hepatocytes, reached a level approximately 7-fold higher than non-parenchymal cells, a figure that is comparable to liposomes modified with LC [68]. Incubation of liposomes with rat primary hepatocytes also indicated that the associated amount of SG-liposomes were almost similar to that of LC-liposomes [69]. From these results, SG-liposomes displayed relatively similar drug delivery efficiencies to galactose-mediated targeting systems.

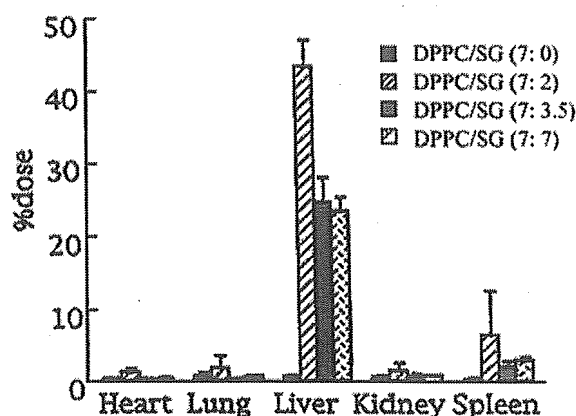


Fig. (4). Biodistribution of calcein entrapped in DPPC/SG liposomes 2 h after an intravenous administration to mice

Each value represents the mean \pm S.D. of three mice [68].

Such specific biodistribution of SG-liposomes may be ascribed to the glucose residue in SG molecules. Hepatic accumulation of liposomes incorporating Ch or SS, was lower than that of SG-liposomes and the distribution SG-liposomes in the liver was mainly in parenchymal cells [6,70]. Glucose residues belonging to SG projects outward from the liposomal membrane and the maximum molar ratio of SG in DPPC membrane was 26 mol% [8]. Further incorporation of SG (DPPC/SG=7/3.5 or 7/7) decreased membrane stability in blood, resulting in less association with hepatocytes [68].

Drug carriers which are unstable in circulating blood would not be able to deliver its drug load efficiently to the targeted site. Increasing the amount of Ch in liposomes makes liposomal membrane more rigid and stable [71,72]. Among some formulations of liposomes with SG, the stability of SG-liposomes in blood was optimal at 30 mol% Ch. Approximately 80% of these SG-liposomes were accumulated in the liver 1 h after intravenous injection [6].

The fluidity of liposomal membranes is a crucial factor for preferential liver uptake. Liposomes incorporating LC and DPPC were taken up by the liver at a higher rate than similar liposomes composed of egg phosphatidylcholine or dimyristoylphosphatidylcholine [58,73]. These reports indicated that the galactose ligand is more suited to receptor-recognition in the rigid liposomal membranes. SG-liposomes having rigid membrane with Ch may augment interactions between SG and hepatocytes.

3.3. Mechanism of SG-Ligands for Liver-Targeting of HepG2 Cells

As mentioned above, SG-liposomes entrapped with calcein were seen to accumulate into the liver at a ratio comparable to LC-modified liposomes [68]. Accumulation of SG-liposomes in the liver may be related to the function of glucose residues in SG. Recognition of glucose residues at the surface of liver cells occurs as a result of glucosylated albumin in ASGP-R exposing positively-charged spacers close to glucose residues. This pathway was seen to be more

effective at uptake than other spacers when presented in murine [63] and isolated rat hepatocytes [74]. Pullulan consists of three α -1,4-linked glucose molecules that are repeatedly polymerized by α -1,6-linkages on the terminal glucose. They were also accumulated in liver parenchymal cells via ASGP-R [75]. Modification at the C-6 position of glucose, rather than C-1 or C-3 positions, allowed specific interactions between ASGP-R in primary murine hepatocytes and glucose-derivatized polyvinylbenzyl polymers [76]. Glucose residue of SG, however, does not have such a spacer and the C-1 position of glucose is connected with a sterol structure.

The liver-targeting efficacy of SG-liposomes was evaluated by means of measuring the associated amount of lipid marker DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) and microspheres with human hepatoblastoma HepG2 cells. These cells have ASGP-R on the plasma membrane [77] and the receptor-mediated association was competitively reduced by co-incubation with AF, which is a desialylated glycoprotein with terminal galactose residues. Uptake of these markers in SG-liposomes into HepG2 cells was inhibited by co-incubation with AF [78] (Fig. 5). This observation suggested that SG-liposomes trigger some interactions with hepatocytes via ASGP-R, or at least an AF-sensitive pathway.

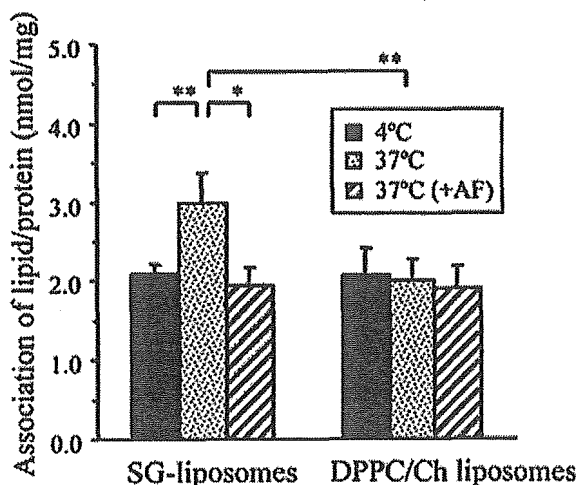


Fig. (5). Association of SG-liposomes with HepG2 cells.

HepG2 cells were incubated for 1 h with SG-liposomes (DPPC/SG/Ch=6/1/3, molar ratio, 100 μ M as DPPC) or DPPC/Ch liposomes (DPPC/Ch=6/4, molar ratio, 100 μ M as DPPC) labeled with 0.4 mol% lipid marker DiI at 4°C or 37°C in the absence or presence of 1 mg/mL asialofetuin (AF). The association was shown in the amount of DPPC.

Each value represents the mean \pm S.D. (n=3) *: p<0.05, **: p<0.01 [78].

The ASGP-R binds glycoproteins that exhibit either β -D-galactose (Gal) or N-acetylgalactosamine (GalNAc) non-reducing terminal residues. At this point, the affinity for GalNAc is approximately 50-fold higher than for Gal, and binding hierarchy is tetraantennary > triantennary >> biantennary >> monoantennary galactosides. This effect is

dependent on the structural organization of the receptor on the cell membrane, because it is not observed on the isolated receptor. In addition to this so-called "cluster effect," some researchers have shown that optimal receptor recognition of synthetic cluster glycosides is also determined by appropriate spacing (at least 15Å) of the sugar residues [79,80]. When SG is positioned onto the surface of liposomes, it may also confer multivalency and appropriate spacing to achieve quantitatively higher affinities, even if these targeting moieties held lower individual affinities.

Interaction of Sit-G with HepG2 cells was also examined by IAsys[®], resonant mirror optical biosensor. Binding of Sit-G to HepG2 cell was seen to increase proportionately with the number of cells added. This reaction was higher than that of human cervical carcinoma HeLa cells, indicating that Sit-G had interacted with hepatocytes [81]. Another specific interaction of SG-liposomes with hepatocyte may be related to their affinity with glucose transporters. Hepatocytes have been found to express GLUT2, a major, facilitative, glucose transporter isoform. This affinity with glucose transporters was utilized for targeting carriers to the liver. A polystyrene-derivative containing glucose moieties was found to interact with GLUT1 from HepG2 cells [82]. Vesicles bearing glucose residues were prepared for targeting GULT to tumours [83]. Preparations of Sit-G NP were found to interact with glucose transporters (SGLT) on intestinal membranes [5]. This data supports the possibility that SG-liposomes interact with hepatic glucose transporters.

Furthermore, efficacy of SG-liposomes as drug delivery carrier was evaluated by means of incubation with FD-4 with HepG2 cells in the presence of Sit-G (Fig. 6). SG and Sit-G, a major component of SG, have been reported to be effective for enhancement of absorption of drugs across mucosal membranes [84]. The polysaccharide FD-4 represents a

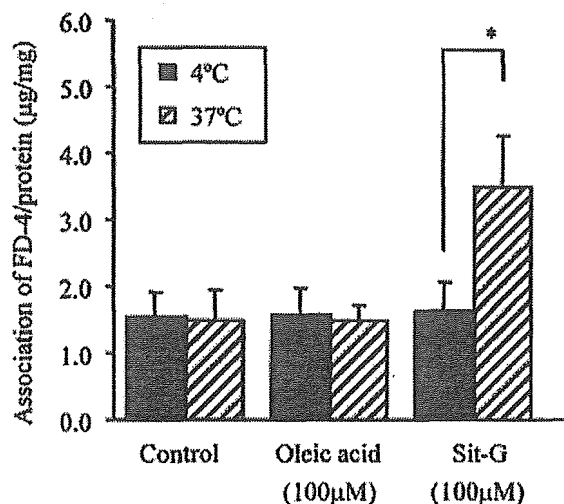


Fig. (6). Association of FD-4 with HepG2 cells enhanced by the addition of Sit-G.

HepG2 cells were incubated for 1 h in FD-4 solution (5 mg/ml) containing 1% DMSO with oleic acid or Sit-G at 4°C or 37°C. FD-4 solution containing 1% DMSO was used as control.

Each value represents the mean \pm S.D. (n=3-4) *: p<0.05 [81].

model of macromolecules that did not associate with HepG2 cells when incubated at 37°C. Application of oleic acid increased drug absorption of dermal [85] and mucosal [86,87] drug delivery systems, although the association of FD-4 was not enhanced by oleic acid in such conditions. Incorporation of Sit-G increased the association of FD-4 in HepG2 cells. This effect was related to the increase in Sit-G concentration and incubation time, as compared with control [81].

Bile salt and glycyrrhizin are known to act as penetration (absorption) enhancers for intestinal [25,88], nasal [12,89] and transdermal [90] absorption of drugs. They also act as ligands for drug delivery to the liver by possessing specific affinities for hepatocytes [91,92]. Bile acids are taken up by normal hepatocytes via sodium-dependent and sodium-independent processes. They act mainly through the sodium-dependent taurocholate co-transporter and covalently linked bile-acid conjugates accumulate in the liver due to their vectoriality [91]. Liposomes modified with glycyrrhizin were effectively accumulated in hepatocytes [92]. Fatty acids, such as oleic acid, possess a high affinity for hepatocytes. Their efficient cellular uptake is the result of a specific interaction with the Fatty Acid Binding Protein (h-FABP) present at the sinusoidal pole of the hepatocyte [93].

Association of FD-4 with HepG2 cells was increased by incubating with Sit-G since Sit-G perturbed lipid membranes. These findings suggested that Sit-G may exhibit a property as both a ligand for liver-targeting and a penetration enhancer at the targeted site, i.e. the liver. After intravenous injection of SG-liposomes, some of them actively transported into hepatocytes and others become passively accumulated in liver macrophages. After the degradation of liposomes by macrophages, the released drug penetrated into hepatocytes with the aid of Sit-G. It has been suggested that SG-liposomes may not only function as liver-targeting drug carrier, but they might also enhance the penetration of drugs in the vicinity of hepatocytes.

3.4. Application of SG-Liposomes in Liver Cancer Chemotherapy

Primary liver cancer, or hepatocellular carcinomas (HCC), is a rare type of cancer in Western countries, but occurs frequently in Africa and Asia. In Japan, HCC is often the sequel to chronic viral hepatitis. More common types of cancer occurring in the liver are metastatic diseases, which originate mainly from primary GI tumours. The prognosis for both HCC and metastases is poor. Treatment of either type of cancer can consist by surgery followed by chemotherapy or radiotherapy.

In cancer chemotherapy, drug targeting can be classified into passive and active targeting. The delivery of drugs to tumours can be realized by entrapping the drug in long-circulating particles. This takes advantage of physiologic factors that improve the localization of particles to target tissues relative to free drug. For example, increased capillary permeability allows passive targeting of particles to solid tumours, known as enhanced permeability and retention effect, especially in tumour or inflammatory sites.

As mentioned earlier, active liver-targeting has been reported using specific ligands, such as galactose residues,

bile acid, glycyrrhizin, and so on. For liver-directed cancer therapy, *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers conjugated with doxorubicin (DXR) have been targeted to hepatocytes by incorporating galactosamine residues. This polymer, known as PK2, was developed as a potential treatment for primary HCC or secondary liver diseases, and is undergoing clinical testing [94,95]. Bile acid-cisplatin complexes, called Bamet, enhanced liver tumour treatment and overcame cisplatin resistance *in vitro* [96,97].

The use of liposomes for the treatment of cancer has been approached from several different directions. The longer the liposomes are able to circulate in the blood, the greater the level of accumulation to the tumour site (passive targeting). This, in fact, is the basis of several different liposomal anthracycline constructs that have given successful results in clinical trials, two of which are on the market. "Active" targeting liposomes with specific ligands, such as antibodies, glycoconjugates, or peptides, have been generally successful *in vitro*, but *in vivo* targeting has been unsuccessful. In tumours, transferrin and folate receptors (FR), and glucose transporters are expressed highly when compared to normal cells.

In a ligand-targeted application for tumours, Lee and Low demonstrated that specific binding and uptake of folate-PEG-liposomes (folate conjugated to the PEG terminus) by a FR expressed in human nasopharyngeal epidermal carcinoma (KB) or HeLa cells [98]. The therapeutic potential of folate-targeted liposomes was initially demonstrated by encapsulating the anticancer drug, DXR [98]. In the past, DNA [99] and oligonucleotides [100] have been formulated with liposomes. There are obviously many other types of macromolecular drug carriers that could be candidates for FR-mediated delivery [101].

Another advantage of particulate formulations is that they can be modified to avoid recognition by efflux pumps, such as P-glycoproteins and multidrug resistance associated protein, which are responsible for "pumping out" lipophilic drug molecules from cells and causing drug tolerance. Use of particulate systems can deliver a P-glycoprotein substrate and antigen across the plasma membrane, allowing them to by-pass P-glycoprotein and lead to intracellular accumulation. Liposomes [102], HPMA copolymer [103] and Pluronic micelles [104] incorporating DXR increased the cytotoxicity of anticancer drugs in cells that over-expressed efflux pumps.

Liver cancer treatment has been attempted with SG-liposomes entrapping DXR (Table 5), pirarubicin (THP) and all-*trans* retinoic acid (ATRA). The drug DXR is one of the most commonly used antitumour agent, but it causes severe adverse effects (e.g. cardiotoxicity, GI disorder and myelosuppression). The drug THP is a derivative of DXR that exhibits fewer adverse reactions and higher antitumour effects than DXR, but distribution in the liver is low. The drug ATRA is a retinoid that induces differentiation in cells and might be suitable for treatment of acute promyelocytic leukaemia. Preparations of SG-liposomes entrapping DXR exhibited increased the antitumour effects of DXR in rats bearing primary liver cancer induced by diethylnitrosamine, and mice bearing M5076 metastatic liver tumours [105].

Table 5. Antitumor Effects of SG-Liposomes Entrapping Doxorubicin (DXR) Against Primary and Metastatic Liver Tumor

Liver tumor	Dosage form	Survival time (day) Mean (S.D.)	% ILS ^{a)}
Rat primary	Saline	25.8 (17.1)	
	Free DXR	19.7 (14.1)	-49.1
	DPPC/Ch liposomes	25.6 (15.5)	-5.5
	SG-liposomes	48.2 (23.3)	92.7
Mouse metastasis (M5076)	Saline	11.4 (1.6)	
	Free DXR	13.1 (3.1)	9.1
	DPPC/Ch liposomes	15.5 (3.9)	27.3
	SG-liposomes	18.2 (10.2)	27.3

^{a)} Percentage increase in life span (% ILS), [(T/C-1)×100], where T and C represent the median survival time of the treated and control animals, respectively. DPPC/Ch liposomes : DPPC/Ch=6/4 (molar ratio), SG-liposomes : DPPC/SG/Ch=6/1/3 (molar ratio) [105]

Accumulation of DXR and THP in the liver by SG-liposomes was 2.4 and 3.7-fold higher than that of free drug, causing an increase in antitumour effects in liver metastatic tumours [105,106]. Preparations of SG-liposomes entrapping ATRA have also shown efficacy against liver metastases [107-109]. These SG-liposomes enhanced the antitumour effects of these drugs owing to a change in drug distribution within the body. These features may correlate with observations of drug accumulation at tumour sites by liposomes modified with SG.

FUTURE DIRECTION

Sterylglucoside has been known for more than 30 years, but the physicochemical properties had not been made clear. We explored the novel physicochemical properties of particulate formulation incorporating Sit-G. Insoluble absorption enhancers can be used effectively in powders, liposomes and nanoparticles. Particulate SG may increase affinity for plasma membranes and reveal novel effects. Absorption enhancement effects of these enhancers have been investigated by direct application onto nasal and intestinal mucosae. However, particulate dosage forms containing enhancers that were delivered into systemic circulation were seen to circulate and passively accumulate in RES and the liver. Active accumulation was thought to have occurred when an interaction was detected between enhancer and hepatocytes, such as in the case of ligand-activated receptor mediation. Thus, Sit-G has been found to fulfil many functions and exert many effects. To study the precise mechanism of absorption enhancers, it might be necessary to find an alternative ligand for targeting of tissues and cells. This appendage would also assist in predicting and quantifying the safe usage of these agents and, perhaps, uncover further novel functions in DDS.

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Design, Synthesis and Gene Delivery Efficiencies of Novel Oligo-Arginine Linked PEG-Lipid: Effect of Oligo-Arginine Length

Masahiko Furuhata¹, Hiroko Kawakami², Kazunori Toma²,
Yoshiyuki Hattori¹, and Yoshie Maitani¹

¹*Institute of Medicinal Chemistry, Hoshi University, Tokyo 142-8501, Japan,*
and ²*The Noguchi Institute, Tokyo 173-0003, Japan*
e-mail: m355@hoshi.ac.jp

Cell-penetrating peptides (CPPs) have the capability of crossing a plasma membrane. They can deliver associated molecules into cells. Oligo-Arg conjugates were demonstrated to have characteristics similar to CPPs in the cell translocation. Four derivatives with various oligo-Arg length (Arg_n-PEG-BDB; n=4, 6, 8, 10) were prepared, and the effect of oligo-Arg length on the gene delivery efficacy was investigated. Arg10-PEG-BDB showed the performance was comparable to LipofectamineTM 2000.

Keywords: cell-penetrating peptide, oligo-arginine, gene delivery.

Introduction

A cellular internalization method using short peptides derived from protein-transduction domains has attracted much attention. Several cell-penetrating peptides (CPPs), such as HIV-1 Tat fragments, less than 30 amino acid residues in length, have the capability of crossing a plasma membrane [1,2]. In addition, they can deliver their associated molecules into cells. Oligo-Arg conjugates were demonstrated to have characteristics similar to CPPs in cell translocation [3-5].

Although investigations delineating the influence of Arg length on the transfection efficiency and uptake of oligo-Args have been reported [3-6], there is no report about oligo-Arg linked poly (ethylene glycol) (PEG) lipids alone as a gene vector. The aim of this study was to design and synthesize simple and effective vectors for use in gene delivery.

In the present study, we synthesized oligo-Arg-lipids of quite different structure from a reported one [6], employing 3,5-bis (dodecyloxy)benzamide (BDB) as the lipid component, and introducing a PEG spacer between the C-terminal of oligo-Arg and the amide group of BDB. Four derivatives with various oligo-Arg lengths were prepared, and the effect of oligo-Arg length on the gene delivery efficacy was investigated in HeLa cells. We demonstrate that the arginine 10-mer exhibits the highest transfection efficiency in HeLa cells among our series of compounds.

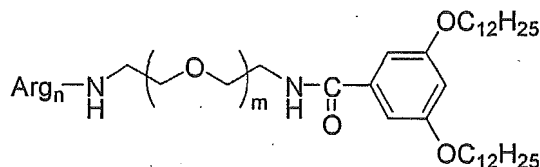


Figure 1. Chemical structures of Arg_n-PEG-BDBs (n=4, 6, 8, 10).

Results and Discussion

We prepared four oligo-Arg-linked lipids of various lengths, Arg4-PEG-BDB, Arg6-PEG-BDB, Arg8-PEG-BDB and Arg10-PEG-BDB (Fig. 1). We evaluated the transfection efficiency of oligo-Arg-PEG-BDB by assaying luciferase activity. Transfection efficiency rose as the number of arginine residues increased. Arg10-PEG-BDB showed the highest level of activity among the oligo-Arg-PEG-BDB derivatives, with about 40-fold, 11-fold and 4-fold higher transfection efficiencies than Arg4-PEG-BDB, Arg6-PEG-BDB and Arg8-PEG-BDB, respectively, on 3h-incubation in serum-free medium. The transfection efficiency in the absence of serum increased about four times as 2 arginine residues were added. Arg10-PEG-BDB showed the highest transfection efficiency, without severe toxicity to cells, and about 1/5 the transfection efficiency of LipofectamineTM 2000, a commercial gene transfection reagent, even in a serum-containing medium. Serum tended to decrease the gene transfection efficiency of oligo-Arg-PEG-BDB, an exception being Arg10-PEG-BDB. These findings well corresponded to the cellular association of the Arg-PEG-BDB/DNA complex determined by flow cytometry. In transfection experiments with the oligo-Arg-PEG-BDB/DNA complex, the arginine residues may be partly used for translocation through the plasma membrane and partly for the formation of complex with plasmid DNA. Therefore, the longer oligo-Arg would be needed for the intracellular delivery of oligo-Arg-PEG-BDB/DNA than the uptake of oligo-Arg-PEG-BDB alone. No severe cytotoxicity of Arg10-PEG-BDB aqueous solution was observed.

Arg10-PEG-BDB is potentially a simple and useful gene delivery tool, because one need only mix it with plasmid DNA and apply the complexes to the cells even in a serum-containing medium.

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