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Original Research Paper

Enhancement of liposomal stability and cellular drug uptake by incorporating tributyrin into celecoxib-loaded liposomes

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ABSTRACT

Tributyrin, a triglyceride analog of short chain fatty acid butyrate, can act as a prodrug of an anticancer agent butyrate. In view of other reports that demonstrated the improved characteristics of conventional liposomes by incorporating small amount of non-phospholipids such as Tween 80, herein we sought to investigate whether the incorporation of tributyrin into the liposomal layers may provide additional advantages for liposomes as an anticancer drug carrier. Liposomes were prepared with dimyristoylphosphatidylcholine as a main phospholipid with or without addition of tributyrin. Celecoxib was loaded in liposomes as a model anticancer drug. Tween 80-incorporated liposomes were also prepared for comparison. Tributyrin-incorporated liposomes were ineffective in enhancing the skin permeation of celecoxib compared to Tween 80-incorporated ones. However, tributyrin-incorporated liposomes enhanced the entrapped celecoxib concentration to an extent comparable to Tween 80-incorporated ones. Furthermore, tributyrin-incorporated liposomes exhibited much higher stability compared to Tween 80-incorporated ones. Finally, the cellular uptake of celecoxib loaded in tributyrin-incorporated liposomes into mouse melanoma cells were more than 10-fold higher compared to that loaded in conventional- and Tween 80-incorporated liposomes. Taken together, the incorporation of tributyrin into conventional liposomes loaded with anticancer drugs may provide an advanced anticancer drug carrier delivering both drug and tributyrin.

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1. Introduction

Histone deacetylases (HDAC) inhibitors that are effective in the transcriptional regulation of aberrant gene expression recently emerged as a potent anticancer agent inhibiting the development and progression of a wide range of cancer [1]. Butyric acid, a naturally occurring short chain fatty acid produced by endogenous intestinal bacterial anaerobic fermentation of dietary fibers [2], is one of the most widely studied HDAC inhibitors. Multiple lines of evidence exist that butyrate is an effective anticancer agent by alone or in combination with other anticancer therapy [3,4]. Despite its capability, butyrate has drawbacks in clinical uses due to its short biological half-life (6 min) [5,6]. In contrast, tributyrin (TB), a triacylglycerol analog of butyric acid (Fig. 1), can act as a prodrug after being cleaved by intracellular lipases and esterases into three molecules of butyric acid [6]. Use of TB was able to overcome the pharmacokinetic drawbacks of butyrate and TB exhibited more potent cancer activities compared with butyrate [7,8]. Furthermore, TB treatment of cancer cells together with other anticancer agents was more effective than single agent treatment, suggesting a carrier delivering both agents may provide an effective anticancer therapy [9].

Liposomes, colloidal vesicular structures composed of lipid layers generated when phospholipids are hydrated in an aqueous media, are one of the most extensively studied drug carriers [10]. In addition to phospholipids, the basic components of liposomal layers, other components are often inserted together in order to provide several advantages as a drug carrier. For example, insertion of cholesterol was proven to improve the rigidity of liposomal membranes, thereby decreasing the leakage of liposomes-entrapped drugs. Inclusion of polyethylene glycol-modified lipids contributed to interfering the liposome recognition by phagocytic cells, thus increasing the *in vivo* half-life of the entrapped substances [11,12]. Recent studies also show that a modified liposome formulation composed of phospholipids and nonphospholipid surfactants exhibit greatly increased skin permeability compared to the conventional liposomes [13]. It was suggested that the membrane flexibility and structural deformability of liposomes were greatly increased due to the existence of bilayer softening components [10,14]. Similarly, inclusion of small amount of oleic acid exhibited increased skin permeability of liposomes [14], providing advantage as a transdermal drug delivery system. The insertion of bile salts in liposomes greatly increased the liposomal stability in the gastrointestinal tract and enhanced the absorption of liposome-incorporated drugs [15]. All of these reports demonstrate that the liposomal characteristics as a drug carrier may be improved [15] by incorporation of certain types of substances in conventional liposomal layers.

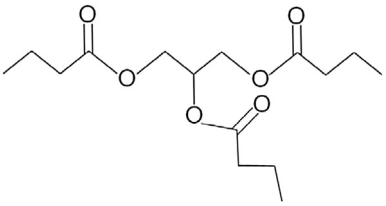

Fig. 1 – Chemical structure of TB.

Hydrophilic drugs are typically encapsulated within the aqueous interior of large unilamellar vesicles [16] whereas hydrophobic drugs are embedded into the phospholipid layers of multilamellar type vesicles to maximize the drug loading concentration [17–19]. Therefore, for hydrophilic drugs, the increase in the volume of aqueous interior may be advantageous to get the enhanced drug loading concentration, whereas for hydrophobic drugs, the increase in the number of phospholipid layers may do. In addition to, either widening the space between phospholipid molecules or between layers may provide more spaces to load the hydrophobic drugs in liposomes. In an attempt to investigate a possibility to develop liposomes containing both of anticancer agent and TB as a more effective anticancer drug carrier, we sought to examine whether the incorporation of small amount (<0.6%, w/v) of TB into liposomal membranes may alter the characteristics of liposomes, including the hydrophobic drug loading capacity. In view of our previous results that demonstrated the cooperative anticancer effects by TB and celecoxib together [9], celecoxib with poor aqueous solubility was selected as a model drug to be incorporated into liposomes.

2. Materials and methods

2.1. Cell lines and cultures

The mouse B16-F10 melanoma cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in DMEM medium (Welgene, Daegu, Korea), respectively. Each medium was supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and 100 units/mL Penicillin/streptomycin. Cells were grown in incubators in a humid atmosphere of 95% air and 5% CO_2.

2.2. Materials

TB and Tween 80 were purchased from Sigma–Aldrich Inc (St. Louis, MO, USA). Celecoxib was obtained from LKT Laboratories (Minneapolis, MN, USA). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were of reagent grade and used without further purification.

2.3. Preparation of liposomes

Liposomes with or without TB were prepared with a slight modification from that described in our earlier study [7]. Briefly, 1 mg celecoxib and 24 mg DMPC with or without TB were first dissolved in t-butyl alcohol. After rapid freezing at −70 °C, mixtures were subjected to freeze-drying (EYELA FDU-1200, Japan). After overnight freeze-drying, the mixture was suspended in 1 ml of 100 mM phosphate-buffered saline (PBS, pH 7.4) to produce a multilamellar type liposomes. Liposomes were briefly vortexed and sonicated in a bath type sonicator for an hour at 37 °C to produce small multilamellar vesicles. To remove the unentrapped/precipitated celecoxib from

celecoxib-incorporated liposomes, the liposome dispersions were immediately filtered through a 0.8 μm membrane filter. The prepared liposomes were stored at 4 °C until use. For comparison, liposomes composed of DMPC and Tween 80 (24:2, weight ratio) were also prepared.

2.4. Characterization of liposomes

To determine the concentration of celecoxib loaded in liposomes, aliquots of liposome dispersions containing celecoxib were freeze-dried, redissolved in 1 ml of the mobile phase and then filtered through a 0.45 μm membrane filter. The amount of celecoxib in the resultant clear filtrate was determined by HPLC analysis as described in the following section.

The mean particle size and polydispersity index (PI) of liposome dispersions were determined by dynamic light scattering method using fiber-optics particle analyzer (FPAR-1000, Otsuka Electronics, Japan). Prior to measurement, dispersions were diluted with filtered PBS. The system was used in the auto-measuring mode. The polydispersity index is a measure of the uniformity of the droplet size distribution in colloidal dispersions [20].

The stability of liposomes was evaluated by monitoring the changes of mean particle size and PI of celecoxib-loaded liposomes during storage at room temperature.

2.5. HPLC analysis of celecoxib

The concentration of celecoxib was analyzed by the HPLC assay as described in our earlier study [7]. Nanospace SI-2 HPLC system (Shiseido, Japan) equipped with a mobile phase delivery pump (Model 3201) and a UV-visible detector (Model 3002), was used. The mobile phase was 45:10:45 (v/v/v) mixtures of acetonitrile, methanol and distilled water containing 0.2% acetic acid (pH 3.5). The injection volume was 10 μl and the flow rate was 1 ml/min through a Lichrosorb RP18 reversed phase column (Phenomenex, Germany). Sample detection was carried out at 254 nm.

2.6. Skin permeation study

Hairless mice (Dae Han Laboratory Animal Research Company, Wonju, Korea) were housed and cared for according to the standards of the Sejong University for animal care and were used under a protocol approved by the Sejong University on animal care. After the hairless mouse was sacrificed by cervical dislocation, the abdominal and dorsal skin was separated surgically. The skin treated with penicillin–streptomycin for the prevention of skin deterioration was mounted between the donor and receptor compartments of a Franz-type vertical diffusion cell with the stratum corneum facing upward. Each cell had a diffusion area of 0.76 cm^2. The receptor chamber was filled with about 5 ml of distilled water containing 4% Tween 80, and was stirred magnetically in a circulating water bath with a constant temperature of 37 °C to keep the skin surface at approximately 33 C. The skin was equilibrated for 24 h with receptor flowing while the donor compartment was dry. About 500 μl of each formulation equivalent to 400 μg of celecoxib were applied into the donor compartment of each cell non-occlusively. At appropriate time intervals up to 30 h, 1 ml of samples were withdrawn from the receptor compartments and replaced by an equal volume of 4% Tween 80 to maintain the receptor volume and sink condition. The experiments were carried out at least in duplicate. Samples were kept at 4 C for a maximum of 2 days until HPLC analysis was performed as described above.

2.7. Cellular uptake of celecoxib

To investigate the effect of liposome incorporation on the cellular uptake of celecoxib, the B16-F10 cells were seeded in 100 cm^2 dishes and cultured for 24 h to allow the attachment of cells. Celecoxib-loaded liposome formulations at a dilution corresponding to 350 μg as celecoxib were added to each dish. At 2 h post-incubation at 37 °C, cells were washed twice with ice-cold PBS to remove unbound liposomes and collected using Trypsin. The obtained cell pellet was lysed by using bioruptor after adding 0.1 ml of 10% sodium dodecyl sulfate. After centrifugation, an equal volume of acetonitrile was added to the cell lysate to precipitate the protein, and 20 μl of supernatant was removed for HPLC analysis. As described above, the concentration of celecoxib was determined from a standard curve and normalized to cellular protein content.

2.8. Statistical analysis

Statistically significant differences between values obtained under different experimental conditions were determined using two-tailed unpaired Student's *t*-tests.

3. Results and discussion

3.1. Effect of TB incorporation on the celecoxib loading capacity of liposomes

To minimize a possibility that the phospholipid emulsions containing TB as an inner oil phase rather than liposomes containing TB as embedded between phospholipids layers can be formed by increasing the content of TB, the content of TB to be added were limited to less than 0.6% of hydration media in the present study. Incorporation of 0.3% TB increased the celecoxib concentration loaded in liposomes by 1.8-fold (Fig. 2). The celecoxib concentration loaded in 0.3% TB-incorporated liposomes was comparable to that in Tween 80-incorporated liposomes (896 ± 34 vs 937 ± 61 μg). However, increase in TB content from 0.3 to 0.6% rather decreased the celecoxib loading concentration (from 896 ± 34 to 833 ± 68 μg). Although the celecoxib solubility in TB was reported to be higher than 100 mg/ml in our earlier study [9], it is unlikely that the increased celecoxib loading in TB-incorporated liposomes is due to the direct solvent effect of TB since no TB dose dependency was observed. Incorporation of both of TB and Tween 80 did not further increase the celecoxib loading concentration compared to that of TB or Tween 80 alone-incorporated liposomes. Therefore, it seems likely that TB incorporation in liposomes affected the loading of celecoxib, a poorly soluble drug, in an indirect manner.

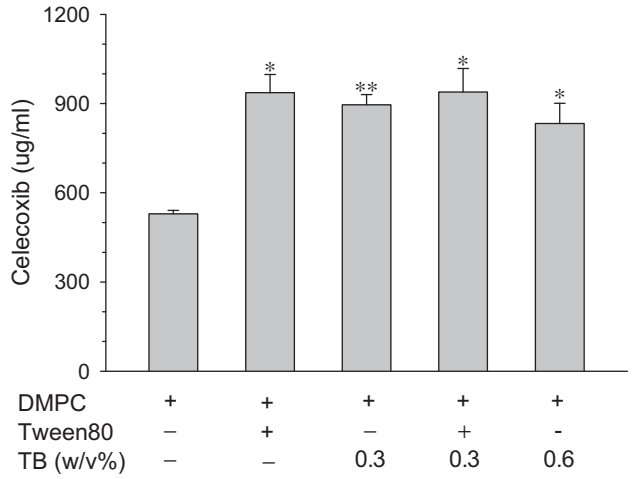

Fig. 2 – Effect of TB incorporation on the concentration of celecoxib loaded in liposomes. The concentration of celecoxib loaded in liposomes was determined by HPLC analysis. Significant differences are indicated by asterisks: *, $P < 0.05$, **, $P < 0.005$, compared with conventional liposomes composed of DMPC alone. Each point represents the mean ± SD ($n = 3$).

3.2. *Effect of TB incorporation on the mean droplet size and physical stability of liposomes*

When the mean particle size and the polydispersity index of celecoxib-loaded liposomes were investigated by dynamic light scattering, it was found that the mean particle size of 0.3% TB-incorporated liposomes was similar to that of conventional liposomes composed of DMPC alone (Table 1). Tween 80-incorporated liposomes exhibited the smallest particle size among various liposomal formulations and the mean particle size of both Tween 80/TB-incorporated liposomes were similar to that of Tween 80-incorporated ones. These data suggest that the reduction of particle size was achieved mainly by Tween 80, but not by TB (Table 1). The polydispersity index of liposomes was lower than 0.3 regardless of liposomal composition, indicating the homogeneity of TB- or Tween 80-incorporated liposomes loaded with celecoxib. Together with the celecoxib loading data, it is presumed that TB was embedded between phospholipid layers, thereby providing more spaces where celecoxib, the hydrophobic drug can occupy.

When liposomes composed of DMPC alone, DMPC/TB, DMPC/Tween 80 and DMPC/TB/Tween 80 were stored at room temperature up to 42 days, the mean particle size of Tween 80-incorporated liposomes gradually increased to more than three fold during storage. In contrast, the mean particle size of both of DMPC liposomes and DMPC/TB liposomes remained unchanged (Fig. 3). The mean particle size of liposomes composed of DMPC/TB/Tween 80 gradually increased during storage but the extent was much lower than that of DMPC/Tween 80 liposomes. Therefore, it appears that the TB incorporation into liposomes did not impair the physical stability of conventional liposomes, whereas the Tween 80 incorporation did. It has been reported that the surfactant-incorporated liposomes, flexible liposomes, are sometimes less stable than conventional ones due to increase in the membrane fluidity [10]. Probably the instability of Tween 80-incorporated liposomes is because Tween 80 is an amphiphilic surfactant that can form other dispersions such as micelles, after being released from liposomal layers during storage.

Table 1 – Mean droplet size and polydispersity index of liposomes prepared with/without incorporation of TB or Tween 80 (Mean ± SD, $n = 3–4$).

Liposome composition	Mean droplet size (nm)	Polydispersity index
DMPC	549 ± 34	0.272 ± 0.081
DMPC/Tween 80	297 ± 26	0.271 ± 0.052
DMPC/TB3	548 ± 22	0.202 ± 0.060
DMPC/Tween 80/TB3	305 ± 22	0.254 ± 0.039

3.3. *Effect of TB incorporation into liposomes on the celecoxib amount penetrated through excised mouse skin*

When the time-dependent changes in the celecoxib concentration penetrated through excised mouse skin were measured during incubation of each liposomal formulation up to 30 h at 37 °C, the concentration of celecoxib permeated tended to be the highest from DMPC/TB/Tween 80 liposomes, followed by DMPC/TB liposomes, and the lowest from DMPC/TB and DMPC liposomes (Fig. 4). Specifically, at 30 h post-incubation with DMPC/TB/Tween 80, DMPC/Tween 80, DMPC/TB or DMPC liposomes, 34, 32, 18 and 18 μg celecoxib was penetrated through 1 cm^2 of skin, respectively. Other studies have demonstrated the increased transport of entrapped drug from Tween 80-incorporated liposomes, in consistent with our data [21]. Therefore, it is concluded that TB incorporation in liposomes is ineffective in enhancing the drug penetration through skin but does not impair Tween 80-aided transport (Fig. 4).

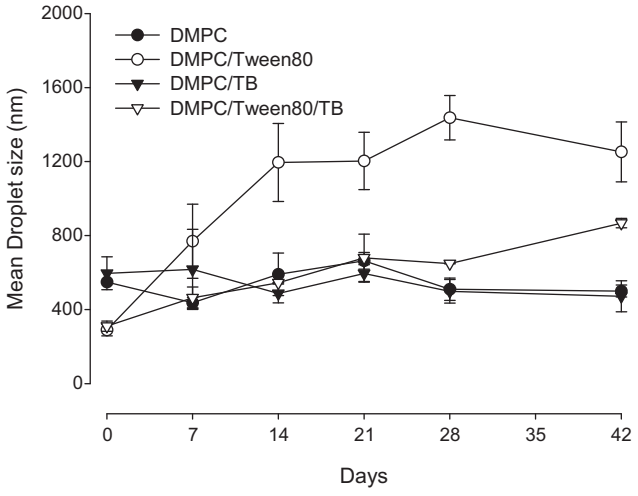

Fig. 3 – Effect of TB incorporation on the changes of mean droplet size of celecoxib-loaded liposomes during storage at room temperature. Each point represents the mean ± SD ($n = 3$).

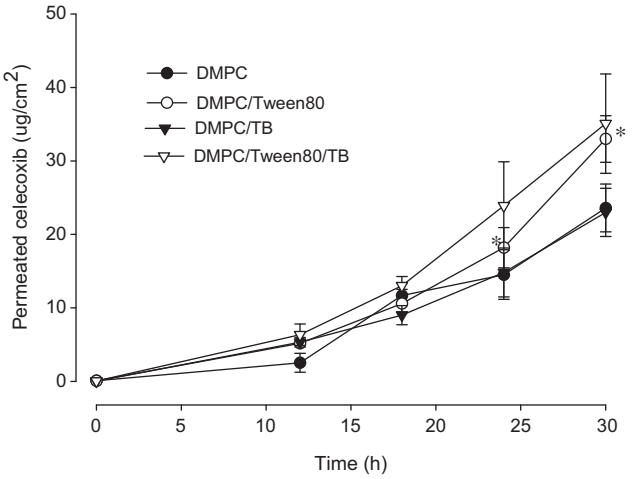

Fig. 4 – Effect of TB incorporation into liposomes on the celecoxib amount penetrated through excised mouse skin. The concentration of celecoxib penetrated through excised skin was determined by HPLC analysis. Each point represents the mean ± SD ($n = 2$). Significant differences are indicated by asterisks: *, $P < 0.05$, **, $P < 0.005$, compared with conventional liposomes composed of DMPC alone.

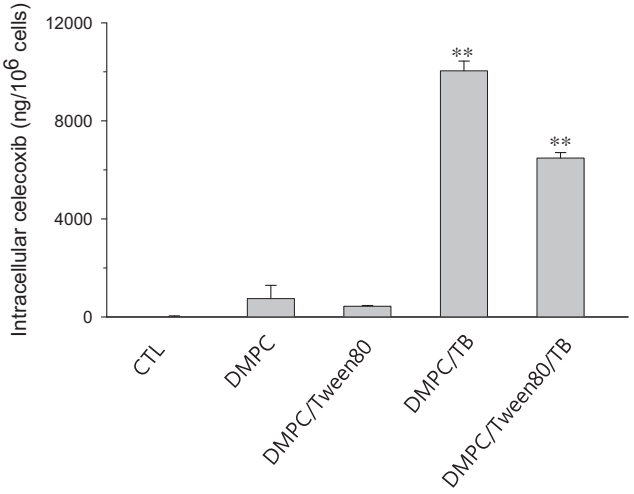

Fig. 5 – Effect of TB incorporation into liposomes on the cellular uptake of celecoxib loaded in various liposomes. Mouse melanoma B16-F10 cells were incubated with each liposome for 4 h. The intracellular concentration of celecoxib taken up by cells was determined by HPLC analysis. Each point represents the mean ± SD ($n = 2$). Significant differences are indicated by asterisks: *, $P < 0.05$, **, $P < 0.005$, compared with conventional liposomes composed of DMPC alone.

3.4. *Effect of TB incorporation on the cellular uptake of celecoxib loaded in liposomes*

Liposome formulation that can deliver higher amount of anticancer drug into cancer cells would be advantageous as an anticancer drug carrier. To further investigate whether the cellular uptake of celecoxib can be altered by the composition of celecoxib-loaded liposomes, the intracellular concentration of celecoxib was compared at 4 h post-incubation of each liposome formulation with mouse B16-F10 melanoma cells. The intracellular concentration of celecoxib after treatment as DMPC/Tween 80- or DMPC-liposome formulation was less than 1000 ng/10^6 cells, suggesting the Tween 80 incorporation into liposomes was ineffective in increasing the cellular uptake of celecoxib (Fig. 5.). In contrast, the uptake of celecoxib loaded in DMPC/TB liposomes was higher than 10,000 ng/10^6 cells, indicating the cellular uptake enhancing effect of TB by more than ten fold compared to conventional liposomes. The uptake of celecoxib loaded in DMPC/TB/Tween 80 liposomes was approximately 6,400 ng/10^6 cells. Our data indicate that TB incorporation into liposomes greatly enhanced the cellular uptake of liposomes whereas Tween 80 incorporation was ineffective. Currently, it remains unclear how TB increased the celecoxib uptake into liposomes. TB is known to exert HDAC inhibitory activity, thereby regulating multiple gene expression. The TB concentration added to cells for the cell uptake experiments was slightly higher than 0.3 mM, a concentration enough to act as a HDAC inhibitor. However, since the incubation period was limited to only 2 h, it is unlikely that the HDAC inhibition by TB already led to the decrease/increase of protein expression involved in endocytosis. Rather, it is more plausible that TB inclusion between phospholipids layers of multilamellar vesicles somehow directly facilitated the endocytosis of liposomes. For example, TB might increase the liposomal fluidity, thereby facilitate the fusion of liposomes with the phospholipids constituting the plasma membrane. Further studies are warranted.

4. Conclusion

Our data demonstrate that small amount of TB incorporation into liposomes enhanced the entrapped celecoxib concentration to an extent comparable to Tween 80-incorporated ones. Furthermore, TB-incorporated liposomes exhibited much higher stability compared to Tween 80-incorporated ones. Although TB-incorporated liposomes were ineffective in enhancing the skin penetration of celecoxib, they increased the cellular uptake of celecoxib into melanoma cells to a significantly higher extent compared to conventional- and Tween 80-incorporated liposomes.

Chemotherapy for cancer patients generally requires combination of drugs differing in the molecular mechanism underlying its anticancer effect to enhance the therapeutic efficacy while minimizing the side effect. TB-incorporated liposomes in the present study may provide an advanced liposomal formulation for the delivery of anticancer drugs and TB simultaneously.

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