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# Dual function of tributyrin emulsion: Solubilization and enhancement of anticancer effect of celecoxib

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ABSTRACT

Tributyrin, a triglyceride analogue of butyrate, can act as a prodrug of an anticancer agent butyrate after being cleaved by intracellular enzymes. We recently demonstrated that the emulsion containing tributyrin as an inner oil phase possesses a potent anticancer activity. Herein we sought to develop tributyrin emulsion as a carrier of celecoxib, a poorly-water soluble drug with anticancer activity. Combined treatment of human HCT116 colon cancer cells with free celecoxib plus tributyrin emulsion inhibited the cellular proliferation more effectively than that of each drug alone, suggesting the possibility of tributyrin emulsion as a potential celecoxib carrier. The mean droplet size of emulsions tended to increase as the tributyrin content in emulsion increases and the concentration of celecoxib loaded in emulsions was affected by tributyrin content and the initial amount of celecoxib, but not by the total amount of surfactant mixture. The concentration of celecoxib required to inhibit the growth of HCT116 and B16-F10 cancer cells by 50% was 2.6- and 3.1-fold lowered by loading celecoxib in tributyrin emulsions, compared with free celecoxib. These data suggest that the anticancer activity of celecoxib was enhanced by loading in tributyrin emulsions, probably due to the solubilization capacity and anticancer activity of tributyrin emulsion.



## 1. Introduction

Acetylation and deacetylation of core nucleosomal histones in cells is regulated by histone acetyltransferases and histone deacetylases (HDAC) (Utley et al., 1998). Aberrant HDAC activity has been associated with the development of certain human cancers. With this regard, 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 (Weisberg et al., 2004).

Butyrate, a naturally occurring short chain fatty acid produced by endogenous intestinal bacterial anaerobic fermentation of dietary fibers (Augenlicht et al., 2002), is one of the most widely studied HDAC inhibitors (Kuwajima et al., 2007). Multiple lines of evidence exist that butyrate is an effective anticancer agent by alone or in combination with other anticancer therapy (Kuwajima et al., 2007; Marks et al., 2000; Munshi et al., 2005). Despite of its capability, butyrate has drawbacks in clinical uses due to its short biological half-life (6 min) (Heerdt et al., 1999; Su and Ho, 2004).

Studies have shown that tributyrin, a triacylglycerol analogue of butyrate, can act as a butyrate of prodrug after being cleaved by intracellular lipases and esterases into three molecules of butyrate (Heerdt et al., 1999). Use of tributyrin was able to overcome the pharmacokinetic drawbacks of butyrate. Furthermore, tributyrin is well tolerated orally and approved for a food additive (Clarke et al., 2001; Hakem et al., 1997; Li et al., 2009). In many studies, tributyrin exhibited more potent cancer activities compared with butyrate (Kang et al., 2011b; Schroder et al., 1998). Since tributyrin is an oil form, it needs to be administered in a suitable dosage form. With this regard, we recently demonstrated a formulation of oil in water type emulsion containing tributyrin as an inner oil phase as a suitable dosage form for tributyrin. Tributyrin emulsion formulated by us inhibited the growth and colony formation of melanoma cells in vitro and in vivo (Kang et al., 2011b). Since inner phase of tributyrin emulsion may provide a space for solubilizing lipid soluble anticancer drugs, anticancer agent-loaded tributyrin emulsion may be an ideal carrier delivering two anticancer agents simultaneously.

Epidemiological and experimental studies have demonstrated that the inhibitors of cyclooxygenase-2 (COX-2), the key enzymes that catalyze the conversion of arachidonic acid to prostaglandins, are effective anticancer agents. Celelcoxib, a selective COX-2 inhibitor, were effective for the prevention and treatment of cancer by alone or in combination with other anticancer agents (Choy and Milas, 2003; Koehne and Dubois, 2004). In addition to COX

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inhibition, celecoxib induced growth inhibition and apoptosis in a wide range of cancer cells, suggesting that its anticancer effects are associated with its growth inhibitory activity. Furthermore, celecoxib functionally blocked p-glycoprotein ((Awara et al., 2004)) and downregulated the expression of MRP1 (Kang et al., 2005), two major drug efflux proteins mediating chemoresistance in cancer cells. Since celecoxib is a highly hydrophobic drug with a poor water solubility of 3–7 μg/ml (Seedher and Bhatia, 2003), numerous attempts have been made to overcome its poor water solubility limitations. For example, celecoxib was loaded in liposomes (Kang et al., 2011a), self-microemulsifying drug delivery systems (Subramanian et al., 2004), microemulsions (Margulis-Goshen et al., 2010) and nanoemulsions (Shakeel et al., 2008). Considering the anticancer efficacy and poor water solubility of celecoxib, a possibility to develop celecoxib-loaded tributyrin emulsion as a potent anticancer drug was investigated in the present study.

## 2. Materials and methods

### 2.1. Cell lines and cultures

The human HCT116 colon cancer cell lines and the mouse B16-F10 melanoma cells were purchased from the ATCC (Manassas, VA, USA) and Korean Cell Line Bank (Seoul, Korea), respectively. HCT116 and B16-F10 cells were maintained in RPMI 1640 medium (Hycolon, Logan, UT, USA) and DMEM medium (Welgene, Daegu, Korea), respectively. Each medium was supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT) 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

Tributyrin, Tween80, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), soybean oil and castor oil 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). Labrafac™ was kindly provided by Gattefosse (NANTERRE Cedex, France). All other chemicals were of reagent grade and used without further purification.

### 2.3. Preparation of emulsions

Tributyrin emulsions were prepared as described in our earlier study (Kang et al., 2011b). Briefly, appropriate weight ratio mixture of tributyrin, DMPC and Tween80 were first dissolved in t-butyl alcohol. After rapid freezing at −70 °C, mixtures were subjected to freeze-drying by freeze dryer (EYELA FDU-1200, Japan). After overnight freeze drying, the mixture was suspended in 100 mM phosphate-buffered saline (PBS, pH 7.4) to give a 10%, w/v tributyrin emulsion. Tributyrin emulsion was briefly vortexed and sonicated in a bath type sonicator for an hour at 37 °C. The prepared emulsions were stored at 4 °C until use.

### 2.4. Size determination of emulsions

The mean droplet size and polydispersity index of emulsions were determined by the dynamic light scattering method using a fiber-optic droplet analyzer (FPAR-1000, Otsuka Electronics, Osaka, Japan). Before measurement, the emulsions were diluted with filtered saline. The system was used in the auto-measuring mode. Droplet size analysis data were evaluated using volume distribution to detect even a few large droplets. The polydispersity index is a measure of the uniformity of the droplet size distribution in colloidal dispersions (Zielinska et al., 2008).

### 2.5. Determination of celecoxib concentration

To determine the solubility of celecoxib in oils, an excess amount of celecoxib was dissolved in various oils and kept at room temperature for 72 h to equilibrate. The samples were centrifuged at 3000 × *g* for 10 min to remove undissolved celecoxib. The supernatant was pulled out, diluted with mobile phase, and then quantified by HPLC analysis as described in our earlier study (Kang et al., 2011a). 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. 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. Cell viability assay

Cells (5–8 × $10^3$/well in 0.1 ml medium) were seeded into 96-well plates. Starting 24 h later, cells were incubated with varying treatments. At 48 h postincubation, the growth and viability of cells were determined by using MTT. The ability of cells to form formazan crystals by active mitochondrial respiration was determined using a microplate reader (Bio-TEK instruments) after dissolving the crystals in DMSO.

### 2.7. Apoptosis ELISA assay

Histone-associated DNA fragments (mono- and oligonucleosomes) produced by apoptosis were quantified by a photometric enzyme immunoassay using Cell Death Detection ELISA$^{plus}$ (Roche Applied Bioscience, USA) following the manufacturer's protocol. Briefly, after treatment, cells were pooled and lysed. Cytoplasmic fractions containing histone/DNA fragments were extracted and adhered to an immobilized anti-histone antibody plate. A peroxidase-conjugated anti-DNA antibody was used for detection of adhered histone/DNA fragments. A colorimetric substrate for peroxidase was then added to each well containing each sample. The net absorbances at the suggested wavelengths ($A_{405\,nm}$ minus $A_{490\,nm}$) were obtained for the lysates derived from treated as well as control cells.

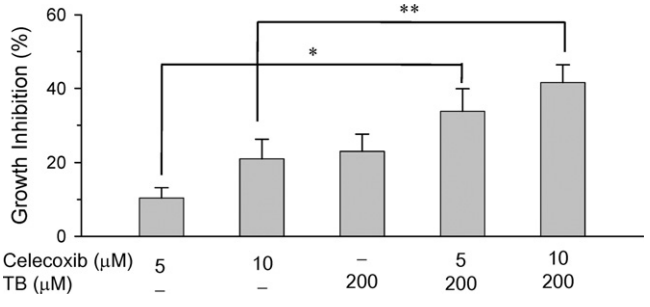


**Fig. 1.** Growth inhibition by free celecoxib, tributyrin emulsion (tributyrin 10%), or celecoxib plus tributyrin emulsion in human HCT116 colon cancer cells. Celecoxib was added as a dimethylsulfoxide solution after dilution with culture media. Results are expressed as percentage growth (mean ± S.D. of triplicate wells) relative to control cells. Significant differences are indicated by asterisks: $^{*}p < 0.05$, $^{**}p < 0.005$, compared with cells treated with celecoxib alone at the same dose. Emulsions were prepared by using 48 mg surfactant mixture composed of 24:23 of Tween80:DMPC.

**Table 1**
Celecoxib solubility in various oils. Celecoxib concentration dissolved in each oil was determined by HPLC analysis.

| Oil | Solubility (mg/ml)[a] |
|---|---|
| Soybean oil | 2.57 ± 0.10 |
| Castor oil | 12.0 ± 0.37 |
| Labrafac | 10.5 ± 0.46 |
| Tributyrin | 101.9 ± 1.53 |

[a] Mean ± SD, $n = 3$–4.

### 2.8. Colony formation assay

500 cells were seeded in 5 cm diameter dishes and incubated for 16 h at 37 °C in an atmosphere containing 5% $CO_2$. Cells were then treated with increasing concentrations of tributyrin emulsion. The culture medium was replaced with fresh medium containing celecoxib-loaded tributyrin emulsion every three days. After 10 days, surviving cells forming visible colonies were counted after staining with 0.5%, w/v crystal violet (Sigma; St. Louis, MO, USA) in 60% methanol.

### 2.9. Statistical analysis

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

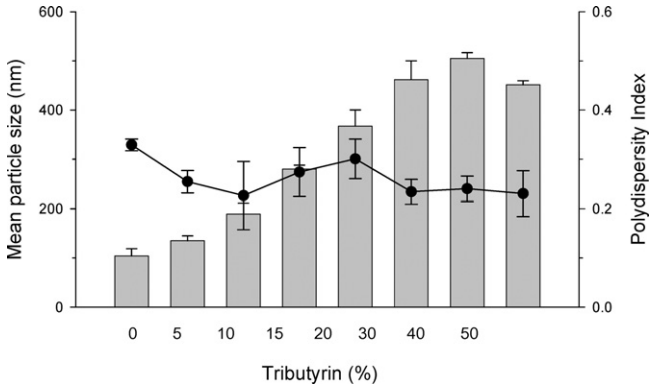


**Fig. 2.** Effect of tributyrin content on the mean droplet size (□) and polydispersity index (●) of emulsions. Emulsions were prepared with 48 mg/ml surfactant mixture composed of 24:23 weight ratio of Tween80:DMPC. Each point represents the mean ± SD ($n = 3$).

## 3. Results

### 3.1. Combined anticancer effect of celecoxib and tributyrin

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. However, combination of anticancer agents may sometimes lead to negative effects, thereby

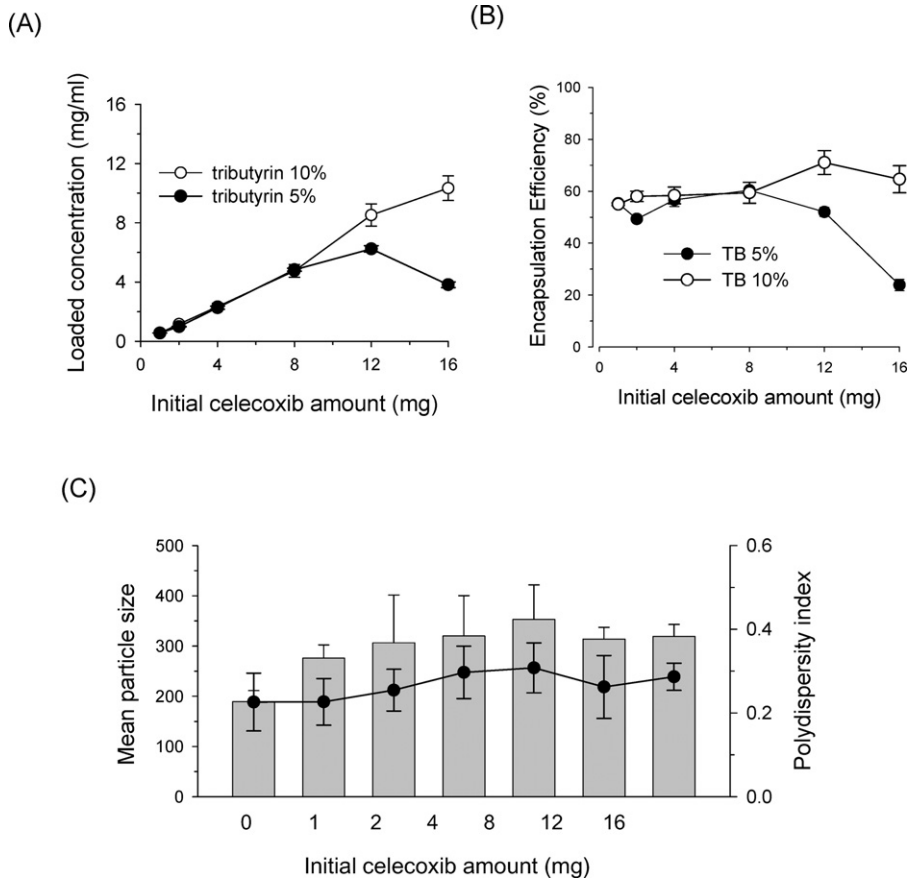


**Fig. 3.** Effect of initial celecoxib amount on the (A) loaded concentration, (B) efficiency and (C) mean droplet size (□) and polydispersity (●) of tributyrin emulsions. Emulsions containing 10% tributyrin was prepared with 48 mg/ml surfactant mixture composed of 24:23 weight ratio of Tween80:DMPC and indicated initial amount of celecoxib. Each point represents the mean ± SD ($n = 3$).

impairing the therapeutic efficacy. To check a possibility to utilize celecoxib-loaded tributyrin emulsion as a potent anticancer agent for combination therapy, we first determined whether combined treatment with celecoxib and tributyrin cooperates to inhibit the proliferation of cancer cells. When the human HCT116 colon cancer cells were incubated with DMSO solution of 5 μM celecoxib, 200 μM free tributyrin or celecoxib plus tributyrin combination, the cellular proliferation was inhibited by 10.4, 22.9 and 33.8%, respectively. Treatment with 10 μM celecoxib, 200 μM tributyrin or celecoxib/tributyrin combination also inhibited the cellular growth by 21.0, 22.9 and 41.6% (Fig. 1). DMSO itself, which was used as vehicle for dissolving free celecoxib, did not affect the cellular growth under our experimental conditions. Therefore, the anticancer effects of celecoxib and tributyrin were additive, suggesting that combined treatment of celecoxib/tributyrin can contribute to improved therapeutic efficacy in cancer treatment.

### 3.2. Solubility of celecoxib in various oils

The high solubility of the drug in the oil phase is important for the oil in water emulsion to maintain the drug in the solubilized form. As a first step to develop tributyrin emulsion as a celecoxib carrier, celecoxib solubility in tributyrin was investigated. For comparison, celecoxib solubility in other oils widely used in pharmaceutical formulations was also determined (Strickley, 2004). Table 1 shows that the solubility of celecoxib was the lowest in soybean oil, modest in castor oil and Labrafac, and the highest in tributyrin. Therefore, celecoxib solubility tended to be higher in short-chain triglycerides (tributyrin) compared to medium-chain (Labrafac®) and long chain (soybean oil, castor oil) (Buyukozturk et al., 2010) triglycerides. In consistent with our data, Earlier study also reported the high solubility of celecoxib in triacetin, another short-chain fatty acid triglycerides (Baboota et al., 2007). Specifically, celecoxib solubility in tributyrin was approximately 39.6-, 8.5- and 9.7-fold higher compared with that in soybean oil, castor oil and Labrafac, respectively. Therefore, tributyrin was a suitable solvent to be incorporated as an inner phase of o/w emulsion for loading celecoxib.

### 3.3. Effect of tributyrin content

Hydrophilic–lipophilic balance (HLB) values of surfactants are important factors for formation of a fine and stable emulsion. Mixing surfactants with high and low HLB generally contribute to the formation of fine and stable emulsion. Considering that Tween80 and DMPC are generally nontoxic and their reported HLB values are 15 and 4 (Buyukozturk et al., 2010), tween80 and DMPC were chosen as surfactants for producing tributyrin emulsion.

Assuming that celecoxib will be incorporated in the inner oil phase, an increased amount of oil phase would contribute to increasing the amount of celecoxib loaded in emulsions. However, the increased oil content in o/w emulsion may often result in unstable emulsions with large droplet size. To investigate the dependency of emulsion size on the content of tributyrin, emulsions containing varying content of tributyrin were prepared and the mean droplet size and the polydispertisy index were measured. Incorporation of tributyrin up to 40% gradually increased the mean droplet size: the mean sizes of emulsions containing 10, 20, 30 and 40% tributyrin were 1.4-, 2.7-, 3.4 and 3.7-fold increased compared with 5% tributyrin emulsion (Fig. 2). Size of emulsions containing tributyrin higher then 40% rather decreased, suggesting that the emulsion size reaches maximum when the tributyrin content was 40%. Since the mean droplet size of lipid injectable emulsions is known to be suitable when less then 500 nm (Driscoll, 2006), it was concluded that the tributyrin content should be less than 40% to be injectable. There was no significant dependency of polydispersity of emulsions on the tributyrin content, suggesting the emulsion homogeneity was not hampered by incorporation of tributyrin up to 50%.

### 3.4. Effect of initial celecoxib amount

Effect of initial celecoxib amount on the loading concentration, the loading efficiency and the resultant emulsion size was investigated as a formulation variable to obtain a tributyrin emulsion in which celecoxib could be loaded at the highest concentration while maintaining small droplet size. For these experiments, the content of tributyrin was fixed at as low as 5 and 10%, considering that the mean droplet sizes of tributyrin emulsions can be increased by entrapping celecoxib.

Celecoxib concentration loaded in 10%-tributyrin emulsion tended to increase according to the increase in the initial celecoxib amount, whereas it increased up to 12 mg initial amount and then rather decreased at 16 mg initial celecoxib amount in tributyrin 5% emulsion (Fig. 3A). It indicates that the inner tributyrin phase of 5%-tributyrin emulsion was saturated with celecoxib at lower initial celecoxib amount because of the lower tributyrin content compared with 10%-tributyrin emulsion. The maximum concentration of celecoxib loaded in 5- and 10%- tributyrin emulsions was 6.3 and 10.4 mg/ml and it was obtained with 12 mg and 16 mg initial celecoxib amount, respectively. These data demonstrate that the loading concentration of celecoxib was dependent on the content of tributyrin incorporated in emulsions, implying that celecoxib was loaded mainly by being dissolved in tributyrin.

As expected from the loading concentration data, the encapsulation efficiency of 10% tributyrin emulsion remained high (>50%) regardless of initial celecoxib amount whereas that of 5% tributyrin emulsion rapidly dropped when the initial celecoxib amount exceed 8 mg (Fig. 3B).

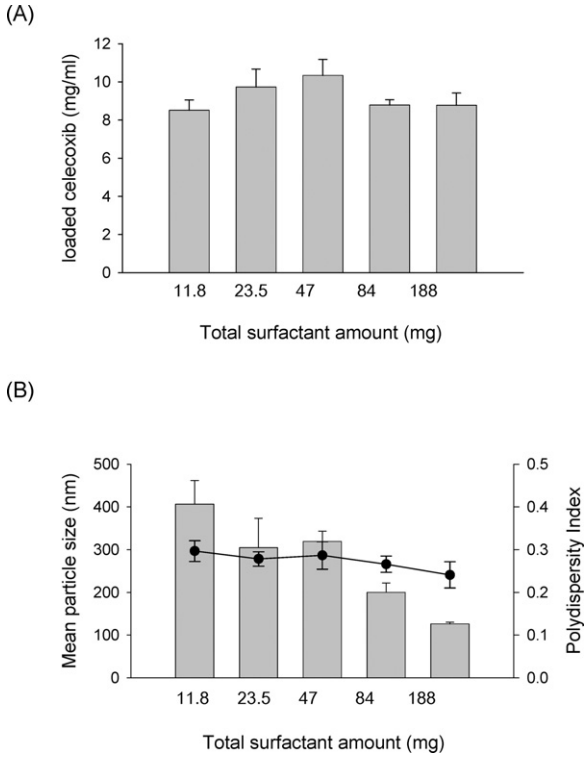


**Fig. 4.** Effect of total amount of surfactant on (A) the concentration of celecoxib loaded in emulsions and (B) the mean droplet size (□) and polydispersity (●) of resultant emulsions. Emulsions containing 10% tributyrin was prepared with 16 mg celecoxib together with indicated total amount of surfactant mixture composed of 24:23 weight ratio of Tween80:DMPC.

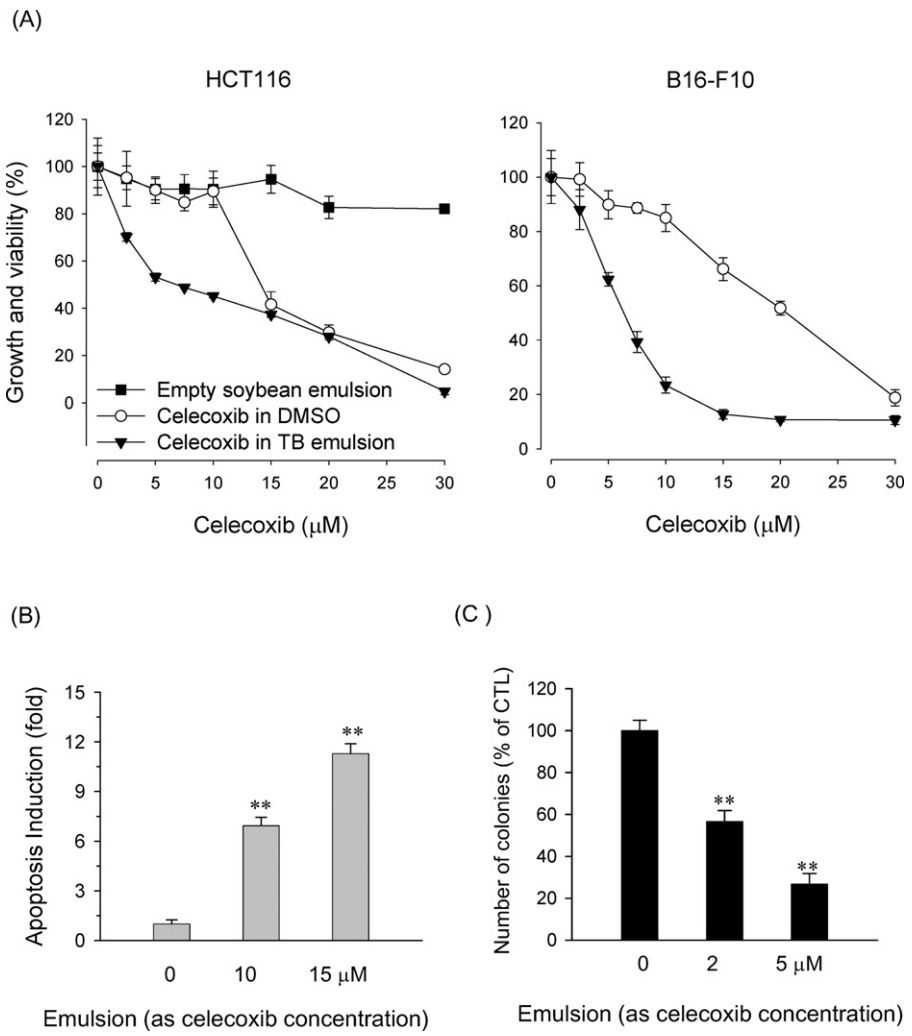


**Fig. 5.** Anticancer efficacy of celecoxib-loaded tributyrin emulsions. (A) Growth inhibition by free celecoxib or celecoxib loaded in 10% tributyrin emulsions in HCT116 and B16-F10 cells. Results are expressed as percentage growth (mean ± S.D. of triplicate wells) relative to control cells. 10% tributyrin emulsions were prepared with 2 mg celecoxib and 48 mg surfactant mixture (24:23 mixture of Tween80:DMPC). (B) Effect of celecoxib-loaded tributyrin emulsion on the apoptosis induction. Prior to determine the apoptosis induction, B16-F10 cells were treated with indicated celecoxib doses of tributyrin emulsion for 36-h. (C) Effect of celecoxib-loaded tributyrin emulsion on the colony formation by cancer cells. The number of colonies in the dish treated with PBS was used as an index for a 100% (control) and this value was used to obtain the percentage colony numbers for other dishes. Data are mean ± SD of at least two independent experiments. Significant differences are indicated by asterisks: $^{**}p < 0.005$, compared with control cells.

Fig. 3C shows that the mean droplet size of 10%-tributyrin emulsion increased by celecoxib loading. It was also affected by the initial celecoxib amount: it gradually increased up to 8 mg of initial celecoxib amount and then remained relatively constant or lower at higher initial celecoxib amount, despite the increased loading concentration (Fig. 3C). The polydispersity index tended to increase slightly according to the increase in the initial celecoxib amount but maintained lower than 0.3 in any formulations regardless of initial celecoxib amount (Fig. 3C).

### 3.5. Effect of amount of surfactant mixture

The loading capacity of o/w emulsions is generally dependent upon the amount of surfactant in addition to the content of oil phase. To investigate the effect of amount of surfactant mixture as an another important formulation variable, 10%-tributyrin emulsion was prepared with 16 mg initial celecoxib amount and increasing amount of surfactant mixture. The concentration of celecoxib loaded in resultant emulsions slightly increased as the amount of surfactant mixture increases from 11.8 to 47 mg but rather decreased at higher surfactant amount (Fig. 4A). In contrast, the mean droplet size of resultant emulsions tended to decrease according to the increase in the amount of surfactant mixture. The mean sizes of emulsions prepared with 23.5, 47, 84 and 188 mg surfactant mixture decreased by 1.3-, 1.3-, 2.0 and 3.2 fold compared with those with 11.8 mg surfactant mixture. The polydispersity index also tended to decrease slightly as the total surfactant amount increases but it maintained below 0.3 regardless of the total surfactant amount (Fig. 4B). It seems likely that the higher amount of surfactant mixture contributed to the formation of smaller and homogeneous emulsions. Taken together, the impact of amount of surfactant mixture on the celecoxib loading capacity was not significantly high but the amount of surfactant mixture was an important factor determining the mean droplet size of emulsions.

### 3.6. Effect of celecoxib loading in tributyrin emulsion on the growth of cancer cells

The growth inhibitory effect of celecoxib loaded in tributyrin emulsions was compared with that of free celecoxib by using two different cancer cell lines. As a control, celecoxib-unloaded (empty)

emulsions containing soybean oil as an inner oil phase instead of tributyrin was prepared. Empty soybean emulsions slightly inhibited the growth of cancer cells but no dose-dependency was found. In contrast, with increasing concentration of celecoxib as free or as tributyrin emulsion-loaded formulation, the growth of both cells decreased in a dose-dependent manner (Fig. 5A). The growth inhibitory effect of celecoxib-loaded tributyrin emulsion was greater than that of free celecoxib solution in both cell lines: the $IC_{50}$ of celecoxib, the concentration of celecoxib required to inhibit the cellular proliferation by 50%, as free or as a tributyrin emulsion-loaded formulation, was approximately 14.0 and 5.3 μM in HCT116 cells, and was 21.0 and 6.7 μM in B16-F10 cells (Fig. 5A). Therefore, the $IC_{50}$ of celecoxib was 2.6- and 3.1-fold lowered in HCT116 and B16-F10 cells by loading celecoxib in tributyrin emulsions. These data suggest that the anticancer activity of celecoxib was improved by loading in tributyrin emulsions, probably due to the solubilization capacity and anticancer activity of tributyrin emulsion.

In consistent with earlier studies that have shown that both celecoxib and tributyrin inhibit the proliferation of cancer cells through apoptosis induction ((Kang et al., 2011b; Venkatesan et al., 2011), celecoxib-loaded tributyrin emulsion induced apoptosis in B16-F10 cells in a dose-dependent manner (Fig. 5B). To further investigate the potential anticancer efficacy of celecoxib-loaded tributyrin emulsion, we also examined whether repeated treatment with celecoxib-loaded tributyrin emulsion could inhibit the colony formation by cancer cells. Repeated treatment with tributyrin emulsion dose-dependently inhibited the colony formation induced by B16-F10 cells (Fig. 5C). These data collectively suggest the potential anticancer efficacy of celecoxib-loaded tributyrin emulsion.

## 4. Conclusion

In the present study, a possibility to develop tributyrin emulsion as a potent carrier for loading anticancer drugs with poor aqueous solubility was explored by loading celecoxib, an anticancer agent with poor water solubility. The loading of celecoxib in tributyrin-based emulsions enabled the solubility limitations of celecoxib to be overcome, as well as to enhance its anticancer efficacy. Therefore, tributyrin-based emulsions served as a drug delivery system with dual functions. Since our data suggest tributyrin-based emulsions as a promising delivery system for anticancer agents with solubility limitations, further studies are warranted to evaluate its in vivo anticancer efficacy.

## Conflict of interest

The authors report no conflict of interest.

## Acknowledgements

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