Letter

Cytotoxicity evaluation of symmetrically branched glycerol trimer in human hepatocellular carcinoma HepG2 cells

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ABSTRACT — An appropriate balance between lipophilicity and hydrophilicity is necessary for pharmaceuticals to achieve fine Absorption, Distribution, Metabolism and Excretion (ADME) properties including absorption and distribution, in particular. We have designed and proposed symmetrically branched oligoglycerols (BGL) as an alternative approach to improve the lipophilic-hydrophilic balance. We have previously shown that stability in circulation and water-solubility of such molecules as proteins, liposomes and hydrophobic compounds are much improved by conjugation to BGL. Albeit these successful applications of BGL, little was known whether BGL could be used in safety. Thus we conducted evaluation of the cytotoxicity of a representative BGL, symmetrically branched glycerol trimer (BGL003) in the cultured cells to clarify its biological safeness. Here we demonstrate that water-solubility of an extremely hydrophobic agent, fenofibrate was more than 2,000-fold improved just by conjugated with BGL003. BGL003 did not exhibit any significant cytotoxicity in human hepatocarcinoma HepG2 cells. Thus BGL003 should be safe and suitable strategy to endow hydrophobic molecules with much hydrophilicity.

Key words: Hydrophilicity, Lipophilicity, Cytotoxicity, Branched oligoglycerols, HepG2, Fenofibrate

INTRODUCTION

Lipophilic-hydrophilic balance is a quite important determinant of Absorption, Distribution, Metabolism and Excretion (ADME) properties of pharmaceuticals. However, it is occasionally a quite tall order to cope with both high pharmacological activities *in vitro* and desired solubility in drug discovery. For that reason, there have been several developed strategies to manage lipophilic-hydrophilic balance. One of the most popular and successful method is conjugating with polyethylene glycol (PEG) group by covalent bond (PEGylation) (Abuchowski and Davis, 1979). It has been successfully applied to several macromolecules such as peptides and proteins including interferons and interleukins, enhancing stability in circulation (Glue *et al.*, 2000; Kita *et al.*, 1990; Knauf *et al.*, 1988). In contrast, it does not seem suitable approach for small molecules possibly because PEGs are so large molecules that it could interfere with bioactivities of the conjugated molecules. Furthermore,

PEGylation may raise cost for the synthesis partly due to its high molecular weight. Highly hydrophilic small molecules comparable to PEG would be more reasonable approach to manipulate lipophilic-hydrophilic balance. Therefore, we have designed symmetrically branched oligoglycerols (BGL) such as the trimer (BGL003) (Fig. 1A), of which molecular weight is as small as 240.25, as alternative highly hydrophilic modular molecules and have shown that stability in circulation and water-solubility of such molecules as proteins, liposomes and hydrophobic compounds were much improved by conjugation to BGL (BGLation) (Ishihara *et al.*, 2010, 2011; Nemoto *et al.*, 1999, 2007a, 2007b, 2010, 2011, 2012a and 2012b; Yamaguchi *et al.*, 2008). Albeit these beneficial effects of BGLation, little was known whether BGL could be used in safety. Thus we evaluated cytotoxicity of the BGL003 using human hepatocarcinoma cells in order to evaluate biological safeness of BGL.

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Fig. 1. Structural formula of BGL003 (A), fenofibrate (B) and FF-BGL (C).

MATERIALS AND METHODS

Materials and Reagents

All reagents were analytic grade and obtained from Wako Pure Chemical (Osaka, Japan) or Kanto Chemical (Tokyo, Japan) unless otherwise stated. Fenofibrate (Fig. 1B) was purchased from Sigma-Aldrich (St. Louis, MO, USA). BGL003 (1,3-bis[(2,2-dimethyl-1,3-dioxan-5-yl)oxy]-2-propanol) and fenofibrate-BGL003 conjugate (FF-BGL) (Fig. 1C) were synthesized as described elsewhere (Hattori *et al.*, 2012; Nemoto *et al.*, 2007b, 2010, 2012c).

Cell culture and cell viability assay

Human hepatocellular carcinoma, HepG2 cells were from American Type Culture Collection (MD, US). Cells were cultured in Dulbecco's Modified Eagle medium containing 10% fetal bovine serum in humidified atmosphere with 5% CO₂. Cell viability was assessed by MTT ((3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with modification (Mosmann, 1983). Briefly, HepG2 cells at 80% confluent in 96-well culture plate were exposed to compounds at the indicated concentration and duration in the culture medium. MTT was added at 1 mg/ml and incubated for 3 hr. Formazan formed in the cells was dissolved in 0.05% Triton X100 with 0.003 M hydrochloric acid and Absorbance at 570/630 nm was measured using a microplate spectrophotometer, infinite M1000, (Tecan, Mannedorf, Switzerland). Cell viability was calculated according to the standard values of wells without cells as 0% and those without compounds as 100%.

Chromatographic analysis by UPLC

Chromatographic analysis was performed by an ultra-high performance liquid chromatography (UPLC) system, ACQUITY (Waters, Milford, MA, USA) with a reversed-phase BEH C18 column (1.7 μ m, 2.1 id x 50 mm length). Aqueous solution of 45% acetonitrile, 0.1% trifluoroacetic acid was used as a mobile phase. Separation was carried out at a constant flow rate at 1.0 ml/min with measuring absorbance at 254 nm.

Evaluation of octanol-water partition coefficient

The octanol-water partition coefficient value (Po/w) was determined according to the standard protocol of Japanese National Institute of Technology and Evaluation based on Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals (OECD, 1995). Briefly, fenofibrate or FF-BGL were solved in water-saturated 1-octanol and vigorously shaken for 2 hr in the same volume of water. Concentration of the compound in each layer separated by centrifuge was determined by UPLC.

Estimation of water-solubility value

Excessive amount of fenofibrate or FF-BGL in water was vigorously sonicated for 5 min at 25 degrees centigrade in ultrasonic bath. Insoluble matters were removed by centrifugation (21,500 x g, for 5 min) and following filtration through 0.45 μ m pores (Millipore, Billerica, MA, USA). Concentrations of compounds in the aqueous solution were determined by UPLC.

Statistical analyses

Values were expressed as means $\pm$ S.E. Comparisons between groups were evaluated by analysis of variance (ANOVA) and Tukey's test. $P < 0.01$ was considered statistically significant.

RESULTS AND DISCUSSION

We have previously shown that BGLation strikingly improved physical properties including hydrophilicity of lipophilic molecules (Ishihara *et al.*, 2010, 2011; Nemoto *et al.*, 2007a, 2011, 2012a, 2012b; Yamaguchi *et al.*, 2008). Recently we succeeded in synthesizing a novel molecule, BGL003-conjugated fenofibrate (FF-BGL) (Fig. 1C) (Nemoto *et al.*, 2012c). Fenofibrate is a popular peroxisome proliferator-activated receptor (PPAR) α agonist clinically used as an anti-hyperlipidemic drug and is known for its exceptionally hydrophobic character. The striking decrease in retention time in chromatographic separation by reversed phase UPLC clearly revealed

Hepatotoxicity evaluation of branched glycerol trimer

Fig. 2. Improvement of hydrophilicity by BGLation. Chromatographic separation of fenofibrate (A) and FF-BGL (B) by reversed phase UPLC. Distribution of the FF-BGL between the same amount of 1-octanol and water, and calculated octanol-water partition coefficient (C). Water-solubility of fenofibrate and FF-BGL (D). Representative data (A, B), $n = 5$ (C, D).

that FF-BGL is far hydrophilic than original fenofibrate (Figs. 2A, B). We next tried to determine Po/w of these compounds, which is a standard strategy to evaluate the lipophilicity-hydrophilicity balance. However, fenofibrate is so hydrophobic that we could not detect it in the water layer by partition with 1-octanol nor determine the Po/w. In contrast, the log Po/w of FF-BGL was measurable and calculated as 1.74 (Fig. 2C). A previous report

using atom/fragment contribution values estimated the log Po/w of fenofibrate as 5.19 (Meylan and Howard, 1995), suggesting that the hydrophilicity of fenofibrate was much improved by BGLation. In accordance, estimated water-solubility values of fenofibrate and FF-BGL were below detectable level (< 0.001 mg/ml) and 2.4 mg/ml, each, clearly demonstrating more than 2,000-times improvement of hydrophilicity of fenofibrate by BGLa-

Fig. 3. Cytotoxicity of BGL003. Viability of HepG2 cells exposed to BGL003 (closed square), glycerol (open circle) or 5-FU (closed triangle) at indicated concentration for 24 hr (A), 48 hr (B) or 72 hr (C). **: $p < 0.01$ vs no treatment (expressed as 100% cell viability), $n = 4$.

tion (Fig. 2D). Fenofibrate is one of the most hydrophobic agents among the pharmaceuticals currently clinically used as the estimated log Po/w of fenofibrate is one of the highest one (Meylan and Howard, 1995). Thus BGLation of fenofibrate may be beneficial to its ADME properties by ameliorating absorption and distribution. We also preliminarily confirmed that lipid-lowering effect of FF-BGL was not impaired using normal rats (Nemoto *et al.*, 2012c). Further study on ADME profiles of FF-BGL is required to clarify comprehensive pharmacological benefit of BGLation of fenofibrate.

Albeit the merits of BGLation on the water-solubility, little was known whether BGL003 could be used safely. Thus we determined cytotoxicity of BGL003 in order to evaluate biological safety of BGL003. We used human hepatocarcinoma HepG2 cells as we expected that this cell line could enable us to simultaneously assess the cytotoxicity of BGL003 and its possible metabolites produced in the human liver. The viability of HepG2 cells were almost unchanged when exposed to BGL003 as well as monomeric glycerol at the concentration of 1~1,000 μM for 24~72 hr (Fig. 3). In contrast, 30, 100 and 1,000 μM of 5-fluorouracil (5-FU) significantly reduced the cells alive since 72 hr after treatments, though it did not show any cytotoxicity within 24 hr of the exposure (Fig. 3). It took a certain time for 5-FU, a typical antimetabolite drug, to exhibit its cytotoxicity assumably because 5-FU needed to be converted into nucleotide form and incorporated DNA synthesis, leading to cell cycle arrest (reviewed in (Pinedo and Peters, 1988)). It is noteworthy that BGL003 did not cause any apparent effects on the cell viability even under such high concentration around submillimolar and so long duration of exposure as the antimetabolite drug to

exhibit cytotoxicity. BGL003 was as innocuous as glycerol which is widely used in miscellaneous commodities around us including foods and medicines. Our study on acute oral toxicity of BGL003 evaluated in normal mice will be reported in the accompanying paper (Miyamoto *et al.*, in press).

There is no way to determine whether BGL is taken up into the cells at the moment. BGL003 is quite difficult to detect because it has no characteristic chemical functional groups but hydroxyl groups nor specific ultraviolet absorption. BGL is thought to be too hydrophilic to easily penetrate plasma membrane. Thus we do not suppose that BGL003 should enter into the cells at the moment. Further study using an isotopic label will provide us much information including all aspects of ADME properties.

In conclusion, we demonstrated that conjugation with BGL003 strikingly ameliorated hydrophobicity of such quite hydrophobic compound as fenofibrate, and that BGL003 did not exhibit apparent toxic effect against the cultured hepatocarcinoma cells. BGL should be safe and suitable alternative to endow hydrophobic molecules with much hydrophilicity.

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