Pharmacokinetic properties of chemical compounds commonly determine their drugabilities to say nothing of their bioactivities. Lipophilic agents easily penetrate lipid bilayers and are distributed to tissues rich in lipids. However, absorbance of hydrophobic chemicals is largely restricted by the unstirred water layer of small intestine, and most of them will be excreted. Furthermore, water-insoluble matters are inactive and not appropriate for injections. It is an important process to manage lipophilic-hydrophilic balance in drug design. Several strategies have been introduced to modulate hydrophilicity of hydrophobic agents. Polyethylene glycol (PEG) group is widely used to ameliorate physical and biological properties of pharmaceuticals (Abuchowski and Davis, 1979). Especially, stability in circulation of such macromolecules as interferons and interleukins are much improved by covalent conjugation to PEG (Glue et al., 2000; Kita et al., 1990; Knauf et al., 1988). However, PEGs are long polymers composed of repeating ethylene glycol units and are usually multiform with various molecular weights, suggesting possibility that not all the PEG-conjugated molecules behave desirably in the same way. PEGs are so large that it could interfere with bioactivities of the conjugated molecules. Therefore, we have designed symmetrically branched oligoglycerols (BGL) such as the trimer (BGL003) (Fig. 1A) as alternative means to endow hydrophobic molecules with much hydrophilicity. We have succeeded in improving the water-solubility of several hydrophobic medicinal small molecules and thermal stability of artificial protein by covalent conjugation to BGL. We have also demonstrated that a representative BGL, symmetrically branched glycerol trimer (BGL003) does not exhibit significant cytotoxicity against human hepatocarcinoma HepG2 cells. However, there have been no reports suggesting whether BGL could be used in safety in vivo. Therefore, evaluation of acute oral toxicity of BGL003 in healthy mice was conducted. Here we demonstrate that an oral administration of BGL003 did not exhibit acute lethal toxicity up to 3,000 mg/kg. Body weight, food intake, blood glucose levels and weights of tissues were not affected by a short-term repetitive administration of increasing doses of BGL003. Biochemical indications related to hepatic disorders and tissue damage were unchanged, either. A single administration study revealed that 50% lethal dose of BGL003 should be more than 2,000 mg/kg. BGL003 will be safe and suitable approach to improve hydrophilicity of hydrophobic compounds.

**Key words:** Hydrophilicity, Lipophilicity, Branched oligoglycerols, Acute oral toxicity, Mice

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**INTRODUCTION**

Pharmacokinetic properties of chemical compounds commonly determine their drugabilities to say nothing of their bioactivities. Lipophilic agents easily penetrate lipid bilayers and are distributed to tissues rich in lipids. However, absorbance of hydrophobic chemicals is largely restricted by the unstirred water layer of small intestine, and most of them will be excreted. Furthermore, water-insoluble matters are inactive and not appropriate for injections. It is an important process to manage lipophilic-hydrophilic balance in drug design. Several strategies have been introduced to modulate hydrophilicity of hydrophobic agents. Polyethylene glycol (PEG) group is widely used to ameliorate physical and biological properties of pharmaceuticals (Abuchowski and Davis, 1979). Especially, stability in circulation of such macromolecules as interferons and interleukins are much improved by covalent conjugation to PEG (Glue et al., 2000; Kita et al., 1990; Knauf et al., 1988). However, PEGs are long polymers composed of repeating ethylene glycol units and are usually multiform with various molecular weights, suggesting possibility that not all the PEG-conjugated molecules behave desirably in the same way. PEGs are so large that it could interfere with bioactivities of the conjugated molecules. Therefore, we have designed symmetrically branched oligoglycerols (BGL) such as the trimer (BGL003) (Fig. 1A) as alterna-
tive small uniform highly hydrophilic modular molecules (Fig. 1B). We have successfully improved stability in circulation and water-solubility of such molecules as proteins, liposomes and hydrophobic compounds by conjugation to BGL to date (Ishihara et al., 2010, 2012; Nemoto et al., 1999, 2007a, 2007b, 2010, 2011, 2012a and 2012b; Yamaguchi et al., 2008). In the accompanying paper, we demonstrated that hydrophilicity and water-solubility of fenofibrate, a quite hydrophobic anti-hyperlipidemic drug (Fig. 1C), was improved more than 2,000-times by conjugation to BGL003 (Fig. 1E) (Miyamoto et al., 2012).

Albeit these benefits of BGL, little was known whether BGL could be used in safety especially in vivo. If functional groups subject to hydrolysis are chosen as a linker conjugating BGL, BGL will be cleft off. Thus we evaluated acute oral toxicity of BGL003 using healthy mice in order to reveal biological safeness of BGL in vivo.

MATERIALS AND METHODS

Materials, Reagents and Animals
All reagents were analytic grade and obtained from Wako Pure Chemical (Osaka, Japan) or Kanto Chemical (Tokyo, Japan) unless otherwise stated. BGL003 (1,3-bis[(2,2-dimethyl-1,3-dioxan-5-yloxy)-2-propanol) was synthesized as described elsewhere (Hattori et al., 2012). BGL003(acetonide), a precursor of BGL003 was exhaustively purified by recrystallization, and the purity of BGL003 was confirmed to be more than 99.9% by ‘H NMR. Ether linkage in BGL003 is stable under biological circumstances unless enzymatically oxidized. We made sure that BGL003 is stable under neutral, basic or weakly acidic conditions of which pH is more than two at least for one year at room temperature. Specific pathogen free grade six-week-old male (for repetitive administration) or seven-week-old female (for determining 50% lethal dose (LD50)) ddY mice were purchased from Japan SLC (Shizuoka, Japan). The mice were individually housed in an animal facility maintained at 22°C with a 12:12-hr light-dark cycle and allowed free access to water and standard rodent chow (DC-8, CLEA Japan, Tokyo, Japan). All the experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, The University of Tokushima.

Study design of drug administration in vivo
We designed the study with referring to the position paper of the Society of Toxicologic Pathology and the current and previous versions of OECD (Organization for Economic Co-operation and Development) guidelines related to acute oral toxicity (OECD, 1987, 2002a, 2002b and 2008; Sellers et al., 2007). Five μl/g body weight of 0.5% carboxymethyl cellulose/saline was used as a vehicle. The mice were randomly divided into three groups, vehicle, BGL003 (n = 6, each) and glycerol (n = 4), at the age of 7 weeks, and orally administered each compound (BGL003 or glycerol) once every other day by gavage at the dose of 30, 100, 300, 1,000 and 3,000 mg/kg body weight as shown in Fig. 2A. Food intake and body weight were monitored daily during the experiment. Blood samples were collected using heparin (Ajinomoto, Tokyo, Japan)-treated syringes 48 hr after the final drug administration (i.e. on the experimental day10) under ether anesthesia with overnight starvation, followed by careful excision and weighing each tissue such as liver, epididymal adipose tissue, spleen, kidney, heart and brain after euthanasia by cervical dislocation. For epididymal adipose tissue or kidneys, only those on the right side were used for the analysis. Tissue weights are expressed as ratios to the body weights.

Biochemical analyses of blood samples
Blood glucose levels were determined once every three days by reflectance glucometer (Roche Diagnostics Japan, Tokyo, Japan) just before drug administration under the ad libitum feeding condition except for fast-
Aminotransferase activities of aspartate transaminase (AST) and alanine transaminase (ALT) in the plasma were examined by a transaminase detection kit (Wako Pure Chemical). Lactate dehydrogenase (LDH) activity in the plasma was determined by LDH detection kit (Wako Pure Chemical) based on the method previously reported (Decker and Lohmann-Matthes, 1988). The activities are expressed as ratios to those from vehicle group because an authentic LDH standard was not available.

Histological analysis
Liver and kidney samples were fixed in 4% paraformaldehyde at 4°C. The samples were dehydrated, embedded in paraffin, cut into 10-μm-thick sections and stained with hematoxylin-eosin. Images were taken by Leica DMD108 microscope.

Evaluation of LD50
LD50 was determined according to the standard protocol of OECD guideline for acute oral toxicity by fixed dose procedure at 2,000 mg/kg body weight using the female mice (n = 5) (OECD, 2002a). We used water as a vehicle (5 μl/g body weight).

Statistical analyses
Values were expressed as means ± S.E. Comparisons between groups were evaluated by Analysis of Variance (ANOVA) and Dunnett’s test. P < 0.05 was considered statistically significant.
RESULTS AND DISCUSSION

Recently we synthesized a novel molecule, BGL003-conjugated fenofibrate, of which water-solubility is much improved (Fig. 1E) (Nemoto et al., 2012c; Miyamoto et al., 2012). We recently noticed that the BGL003-conjugated fenofibrate yielded an active form of fenofibrate, fenofibric acid (Fig. 1D) when administered in vivo, suggesting that BGL003 should be easily applicable to pro-drug (Nemoto et al., 2012c). However, the safety of BGL003 will concern its success in future clinical development as BGL003 should be cleaved in vivo.

This is the first report evaluating toxicity or safety of BGL in vivo. Our major object of this study is to clarify whether BGL003 exhibits acute toxicity in vivo or not. However, we chose to repeatedly administer increasing doses of compounds to the same mice for a short term in addition to a study by a single bout. The compounds were evaluated up to 3,000 mg/kg body weight as the current OECD guidelines require the study using a dosage more than 2,000 mg/kg body weight only when necessary (OECD, 2002a, 2002b). The oral administration was chosen although it is uncertain whether BGL003 would penetrate well into blood stream, because it is a major route of drug administration and gastric acidic ambiance was thought to be a possible reason of hydrolysis of BGL conjugated compounds. This study design enables us to simultaneously estimate acute and short-term accumulative toxicities, though it will be difficult to attribute observed phenomena to whether results of accumulative effects or the last single dosage.

Changes in body weight are widely recognized as an indication of the overall health in vivo. Oral administration of BGL003 as well as glycerol did not affect body weight of normal healthy mice (Fig. 2B). Six mice were used for BGL003 group, and none of them died during the study. Daily food intake was increased 19% in BGL003 group only during the experimental day 3-4 (Fig. 2C). However, food intake during the experimental day 2-4, i.e. for 48 hr after administration at 100 mg/kg body weight, was not affected by BGL003 (vehicle, 11.7 ± 0.48; BGL003, 12.9 ± 0.52; glycerol, 12.0 ± 0.64; (g/48 hr)), nor were the cumulative food consumptions during the study different (vehicle, 54.9 ± 2.8; BGL003, 57.0 ± 1.9; glycerol, 52.6 ± 3.0 (g)). Considering these results, it is supposed that BGL003 does not affect feeding behavior or body weight. These data also clearly indicate its adequate tolerability in vivo.

Even though it is uncertain whether BGL003 is metabolized or excreted as it is in vivo at the moment, we compared the effects of BGL003 with those of monomeric glycerol since the structurally possible intermedi-

| Table 1. Effects of acute repetitive oral administration of BGL003 on tissue weights |
|---------------------------------|----------------|----------------|
|                                 | vehicle        | BGL003         | glycerol       |
| Adipose tissue (g/g body weight)| 0.0071 ± 0.0015| 0.0076 ± 0.0015| 0.0065 ± 0.0008|
| Liver (g/g body weight)         | 0.040 ± 0.001  | 0.042 ± 0.001  | 0.040 ± 0.001  |
| Heart (g/g body weight)         | 0.0047 ± 0.002 | 0.0042 ± 0.0001| 0.0044 ± 0.0002|
| Kidney (g/g body weight)        | 0.0080 ± 0.0002| 0.0072 ± 0.0004| 0.0078 ± 0.0004|
| Brain (g/g body weight)         | 0.013 ± 0.001  | 0.013 ± 0.000  | 0.014 ± 0.000  |
| Spleen (g/g body weight)        | 0.0025 ± 0.0001| 0.0027 ± 0.0002| 0.0026 ± 0.0003|

n = 6 (vehicle, BGL003), n = 4 (glycerol)
No data show significant difference from those from vehicle group.

| Table 2. Effects of acute repetitive oral administration of BGL003 on biochemical indexes |
|---------------------------------|----------------|----------------|
|                                 | vehicle        | BGL003         | glycerol       |
| Fasting Blood Glucose (mg/dl)   | 78.3 ± 4.8     | 83.5 ± 1.7     | 76.8 ± 4.9     |
| AST (Karmen Unit)               | 114.5 ± 19.2   | 105.0 ± 9.4    | 79.4 ± 8.3     |
| ALT (Karmen Unit)               | 19.9 ± 2.9     | 21.5 ± 1.4     | 19.0 ± 0.8     |
| LDH (ratio to vehicle)          | 1.00 ± 0.11    | 1.21 ± 0.14    | 0.74 ± 0.75    |

n = 6 (vehicle, BGL003), n = 4 (glycerol)
No data show significant difference from those from vehicle group.
ate of BGL003 would be glycerol if metabolized. Glycerol is primarily produced by lipolysis in adipose tissue, and used as a substrate for gluconeogenesis in liver under physiological condition. Thus we also monitored changes in blood glucose levels, but they were not influenced by BGL003 or glycerol treatments except for a slight increase at the experimental day 6 in glycerol group (Fig. 2D).

Weights of tissues are quite simple but provide us one of the most important toxicological and pharmacological information (Michael et al., 2007; Peters and Boyd, 1966; Sellers et al., 2007). No significant differences in the weights of tissues such as epididymal fat pad, which is a representative visceral fat, liver, heart, kidney, brain and spleen among the groups were observed (Table 1). BGL003 is not supposed to exhibit significant toxicity on the metabolic and circulatory tissues, central nervous and immune systems. Furthermore, the liver and kidney exhibited no apparent histologically toxic symptoms such as ectopic lipid accumulation, fibrosis or infiltration of leukocytes by BGL003 or glycerol (Fig. 3).

We next examined effects of the repetitive administration of increasing amounts of BGL003 on blood biochemical indexes. Fasting blood glucose levels were about the
same among the groups albeit glycerol is a potent substrate of gluconeogenesis as aforementioned (Table 2). Although it may be simply due to the long duration of 48 hr from the last administration, our data suggested that the effects of BGL003 on whole-body glucose metabolism are limited even if they exist. It is difficult to predict when the best timing to detect the effects of drug is. We gave priority to evaluating acute lethal toxicity, and decided to administrate every other day and to analyze tissues 48 hr after the last dosage. AST and ALT activities in the plasma, which are widely accepted as golden standards for evaluating hepatotoxicity, were not increased by BGL003 treatments. In addition, LDH activities in the plasma, reflecting damage on the tissues such as liver, skeletal and cardiac muscles and red blood cells, were not elevated by BGL003 (Table 2). These results demonstrated that acute toxicities on tissues including hepatotoxicity of BGL003 should be as low risk causing as glycerol, which is widely used in miscellaneous commodities around us including foods and medicines, if it exists. It is quite consistent with our result in the accompanying paper that BGL003 did not show significant cytototoxicity against human hepatocarcinoma HepG2 cells (Miyamoto et al., 2012).

Our data above suggest adequate tolerability of BGL003 in vivo. We furthermore conducted a single-dose administration study to determine LD50 in compliance with the OECD standard protocol, and confirmed that LD50 of BGL003 is estimated to be more than 2,000 mg/kg body weight (OECD, 2002a). BGL is expected to be conjugated to bioactive molecules when taken as a component of a drug. Thus an assumed substantial amount of BGL003 will not usually exceed 1,000 mg/kg body weight since the molecular weight of BGL003 is as small as 240.25, and therefore, it is suggested that BGL003 should be innocuous enough even if used as pro-drugs. Potential risks and hazards of chemical compounds were universally classified and labeled by Globally Harmonized System of Classification and Labeling of Chemicals (GHS) in United Nations. BGL003 is suggested to be as safe as chemicals classified in hazard category 5, in which LD50 is estimated more than 2,000 mg/kg body weight since the molecular weight of BGL003 is estimated to be more than 2,000 mg/kg body weight (OECD, 2002a). BGL will be a safe and suitable alternative to endow hydrophobic molecules with much hydrophilicity.

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REFERENCES


Acute toxicity evaluation of branched glycerol trimer in mice


