Original Article

Gamma Delta Tocotrienols Reduce Hepatic Triglyceride Synthesis and VLDL Secretion

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***Aim*: Present study aimed to elucidate the suppression of serum lipids by gamma- and delta-tocotrienol ($\gamma\delta$T3).**
***Methods*: The lipid-lowering effects of $\gamma\delta$T3 were investigated using HepG2 liver cell line, hypercholesterolemic mice and borderline-high cholesterol patients.**
***Results*: *In-vitro* results demonstrated two modes of action. First, $\gamma\delta$T3 suppressed the upstream regulators of lipid homeostasis genes (DGAT2, APOB100, SREBP1/2 and HMGCR) leading to the suppression of triglycerides, cholesterol and VLDL biosyntheses. Second, $\gamma\delta$T3 enhanced LDL efflux through induction of LDL receptor (LDLr) expression. Treatment of LDLr-deficient mice with 1 mg/day (50 mg/kg/day) $\gamma\delta$T3 for one-month showed 28%, 19% reduction in cholesterol and triglyceride levels respectively, whereas HDL level was unaltered. The lipid-lowering effects were not affected by alpha-tocopherol (αTP). In a placebo-controlled human trial using 120 mg/day $\gamma\delta$T3, only serum triglycerides were lowered by 28% followed by concomitant reduction in the triglyceride-rich VLDL and chylomicrons. In contrast, total cholesterol, LDL and HDL remained unchanged in treated and placebo groups. The discrepancies between *in-vitro*, *in-vivo* and human studies may be attributed to the differential rates of post-absorptive $\gamma\delta$T3 degradation and LDL metabolism.**
***Conclusion*: Reduction in triglycerides synthesis and transport may be the primary benefit caused by ingesting $\gamma\delta$T3 in human.**

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Key words; Tocotrienol, Vitamin E, Triglycerides, Cholesterol, Cardiovascular, Antioxidant

Introduction

In 1922, embryologist Evans discovered tocopherols (TP), which are needed for human reproduction[1, 2]. More than 40 years later, isolation of tocotrienol (T3) from latex was first reported by Morton[3]. To date, vitamin E has been found to consist of T3 and TP, which provide a significant source of anti-oxidant activity in all living cells[4]. This common anti-oxidant attribute reflects the similarity in the chemical structures of T3 and TP, which differ only in their structural side chain (contains farnesyl for T3 or saturated phytyl side chain for TP). Historically, natural products have been a rich sources of biologically active compounds for drug discovery[5]. T3 is an important plant vitamin E constituent. In contrast to corn, wheat, and soybean oils, palm and rice bran oils contain >45% T3, which consists of four isomeric forms: alpha (α), beta (β), gamma (γ), and delta (δ). Apart from its anti-oxidant activity, T3 has been shown to

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possess anti-cholesterol[6] and anti-cancer activities[7].

Cholesterol and triglycerides are two forms of lipid. Both cholesterol and triglycerides are necessary for life because cholesterol is required for building cell membranes and making several essential hormones[8] whereas triglycerides provide much of the energy needed for cells to function[9]. Physiologically, they can not dissolve and circulate in the blood without combining with lipoproteins (VLDL, LDL, HDL and chylomicrons). Apart from chylomicrons, which transport primarily triglycerides from the exogenous pathway (intestinal absorption), LDL/HDL and VLDL are the major lipoproteins responsible for carrying cholesterol and triglycerides from the endogenous pathway (hepatic biosynthesis) respectively[10].

In the past three decades, the lipid-lowering properties of T3 have been demonstrated in cell lines[6], animal models[11] and humans[12], focusing primarily on the reduction of hepatic cholesterol biosynthesis. The *in vitro* mechanism may involve a reduced 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) protein synthesis rate and an increased degradation rate, as found in hepatoma HepG2 cells[13]. The different T3 isomers possess various degrees of cholesterol-lowering activity. γδT3 is claimed to be more active than αT3 in inhibiting HMGCR, whereas βT3 has a slight effect[14]. In contrast, αTP induces HMGCR activity[15].

Clinical findings concerning the effects of T3 on circulating cholesterol, triglycerides and lipoproteins in humans are inconsistent. The four earlier studies published by Tan *et al.*[16] and Qureshi *et al.*[12, 17] showed lipid-lowering effects (total cholesterol, LDL and triglyceride reduction) of the T3-rich fraction (TRF) from palm oil. In these studies, no changes were found in serum HDL cholesterol concentration.

In contrast to the positive studies, several research groups failed to observe significant changes in the serum lipid and lipoprotein profiles after supplementation with TRF. In the study by Wahlqvist[18], hypercholesterolemic patients who used supplements containing TRF in increasing doses from 60−240 mg/day for 20 weeks did not show an improved lipid profile. These findings were confirmed by Tomeo[4], who examined the TRF effects in increasing doses from 224−336 mg/day for 18 months on serum lipids and lipoproteins in hyperlipidemic men and women with carotid atherosclerosis. Furthermore, Mensink[19] detected no changes in serum lipid and lipoprotein concentrations in mildly hypercholesterolemic men who received supplements containing TRF consisting of 135 mg/day of T3 for 6 weeks. O'Byrne[20] supplemented hypercholesterolemic patients with 250 mg/day of α, γ, or δ-tocotrienyl acetates from palm oil for 8 weeks in addition to an AHA step I diet. Serum lipid and lipoproteins were also unaffected.

Given the controversial findings from previous *in vitro*, *in vivo* and human studies, we aimed to evaluate the lipid-lowering effects of γδT3 in the liver cell line HepG2, and hypercholesterolemic mice and patients. In addition, this study aimed to investigate whether high αTP (50%) co-supplementation with γδT3 will be less effective than purified γδT3 supplementation. Finally, a 8-week hypercholeterolemic human trial with 120 mg/day γδT3 supplementation was conducted in Japan to evaluate the impact of the two most potent T3 isomers on serum cholesterol, triglycerides, LDL-cholesterol, and HDL-cholesterol. Since LDL particles vary in size/density, and studies have shown that small-dense LDL (sd-LDL) particles equate to a higher risk factor for coronary heart disease than larger and less dense LDL particles[21], we further evaluated whether γδT3 supplementation impacted the 20 fractions of triglycerides and cholesterol lipoproteins of various size/density[22].

Materials and Methods

Cell Line, Culture Conditions and Chemicals

Human hepatocellular carcinoma cells (HepG2) (ATCC, Rockville, MD) were maintained in RPMI 1640 with L-glutamine and 25 mM HEPES (E15-842; PAA Laboratories GmbH, Austria) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin at 37℃ in 5% CO_2. HepG2 cells were prepared for treatment (day 0) in 10% FBS-supplemented media. On day 1, the cells were pre-treated in media which had the FBS replaced with 10% lipoprotein-depleted serum (LPDS) (BioWest, France), and supplemented with 50 μM mevastatin (Sigma Aldrich, St Louis, MO) plus 50 μM mevalonic acid lactone (Sigma Aldrich). After 16h, the cells were treated with 5 μM 25-hydroxycholesterol (25-HC) (Sigma Aldrich), 5 μM simvastatin (ST) (Sigma Aldrich), 20 μM γ-, δT3 (Davos Life Science, Singapore), and 20 μM γδT3 (Davos Life Science) prepared in fresh media containing 50 μM mevastatin and 10 μM mevalonic acid lactone (Sigma Aldrich). Cells were harvested after 16 h for analyses. T3 and TP isomers were purified from palm oil using Davos separation technology. Crude palm oil feed was purchased from KLK Berhad. Using the corresponding VE isomers as a reference standard, the purity was verified as ≥97% by the HPLC percentage area. Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich.

Cell Viability Study

For cell viability study, 5×10^3 HepG2 cells resuspended in 100 μL medium were plated in each well of a 96-well plate. The cells were then treated with different concentrations (20, 40, 80 μM) of T3 isomers for 24 h. After treatment, 20 μL MTT solution was added to each well and the cells were incubated at 37℃ for 2 h. Formazan crystals were then re-suspended in 100 μL DMSO and the intensity at 595 nm was measured. Each experiment was repeated three times in triplicate and the growth curves showed the means and standard deviations.

T3 Extraction from Serum

Sera were thawed and sonicated in an ultrasonic bath (Lab Companion, Vernon Hills, IL) for 5 min, followed by vortexing for 10 sec. Then, 100 μL serum was transferred into an IWAKI Pyrex glass tube (Jawa Tengah, Indonesia) containing 900 μL water, and 5 μL δT3 of 99% purity (100 mg δT3 dissolved in 1 mL ethanol) was used as an internal standard solution and added to the mixture. The tube was vortexed for 10sec and sonicated for 2 min. A 4 mL sample of butylated hydroxytoluene (BHT; Sigma Aldrich) solution (5 mg BHT in 100 mL heptane) was added to the tube to minimize the oxidation of target analytes. Liquid-liquid extraction was performed by vortexing vigorously for 10 sec. After liquid-liquid extraction, the tubes were centrifuged at 3450 xg for 5 min in Heraeus Multifuge 3-SR Centrifuge (Newport Pagnell, Buckinghamshire). A 3.9 mL sample of the organic layer was transferred into another Pyrex tube. The extraction was repeated and the second organic layer was removed and pooled with the first layer. The organic solution was evaporated using a Buchi rotavapor R-205 (Flawil, Switzerland), and the dried residue was reconstituted in 1.5 mL heptane and filtered, followed by HPLC analysis

Western Blotting

Cell lysates were prepared by suspending cell pellets in lysis buffer [50 mM Tris-HCl (pH8.0), 150 mM sodium chloride, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mg/mL aprotinin, 1 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride]. Protein concentration was measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA). An equal amount of protein (30 μg) was loaded onto a 10% SDS polyacrylamide gel for electrophoresis and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham, Piscataway, NJ). The membrane was then probed against HMGCR (H-300) (Santa Cruz Biotechnology, Santa Cruz CA), sterol regulatory element binding proteins 1/2 (SREBP-1/2) (BD Biosciences, Pharmingen, USA), LDL receptor (LDLr) (EP1553Y) (Novus Biologicals, CO), and PPARα (H-98), APO100 and diacylglycerol O-acyltransferase 2 (DGAT2) (H70) (Santa Cruz Biotechnology). The expression of β-actin (I-19) (Santa Cruz Biotechnology) was assessed as a loading control for total cell lysates. After incubation with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), signals were detected by the ECL Wstern blotting system (Perkin Elmer, Waltham, MA).

Real-Time Polymerase Chain Reaction

Total RNA was isolated from the treated cells using the Promega SV Total RNA Isolation system (Promega, USA). The cDNA was synthesized from 1 μg total RNA using the Promega ImProm-II Reverse Transcription System (Promega). Real-time PCR was carried out using the ABI PRISM® Sequence Detection System (Applied Biosystems, USA) according to the manufacturer's protocol for TaqMan® Gene Expression Assays (Applied Biosystems). A standardized amount of 100 ng cDNA was used per PCR reaction. The PCR procedure was performed with specific TaqMan® Probe sequences (HMGCR: 5'to3' TGGTA CCATG TCAGG GGTAC GTCAG; GAPDH: 5'to3' GGCGC CTGGT CACCA GGGCT GCTTT) linked to a reporter dye, 6-FAM, at the 5' end. Cycle parameters on the 7900HT Sequence Detection System were according to the recommended standard thermal cycler protocol: 40 cycles of 95℃ for 15 sec and 60℃ for 1 min. Each PCR reaction was performed at least in duplicate and the level of each gene expression was determined relative to the normalized GAPDH gene.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The same source of synthesized cDNA was used for RT-PCR. A standardized amount of 100 ng cDNA was used per PCR reaction. The PCR procedure was performed with specific human primer pair sequences (GAPDH: 5'to3'F ATGAC ATCAA GAAGG TGGTG; 5'to3'R CATAC CAGGA AATGA GCTTG; SREBP1: 5'to3'F TGCTG ACCGA CATCG AAGAC; 5'to3'R CTGTC TTGGT TGTTG ATAAG CTGAA; SREBP2: 5'to3'F GGCGA TGGAC GACAG CGGCG GCT; 5'to3'R GTCAG TCTGG CTCAT CTTTG ACCTT; Apo B48: 5'to3' F CTGCA AATGA GACAC TTTCT C; 5'to3'R TGCTC TCATC AAAGG CATGA; Apo B100: 5' to3'F GCAAG GAGCA ACACC TCTTC; 5'to3'R TAATA CGACT CACTA TAGGG AGGTC CACAC TGAAC CAAGG TC; Apo C3: 5'to3'F ATGCA

CTGAG CAGCG; 5'to3'R ACGGC TGAAG TTGGT; Apo E: 5'to3'F ACCCA GGAAC TGAGG GC; 5'to3'R CTCCT TGGAC AGCCG TG; DGAT2: 5'to3'F AGTGG CAATG CTATC ATCAT CGT; 5'to3'R AAGGA ATAAG TGGGA ACCAG ATCA). Cycle parameters were as follows: 95℃ for 10 min, 40 cycles of 94℃ for 30 sec, 60℃ for 1 min and 72℃ for 2 min, and 72℃ for 10 min. GAPDH expression was assessed as the loading control.

***In vivo* Hypercholesterolemic Mice**

The experimental protocol was approved by the IACUC committee of the A-STAR Biological Resource Centre (BRC) at Biopolis (IACUC no.: 080302). Researchers involved in this study passed a course on, "Responsible care and use of laboratory animals (RCULA)". Hypercholesterolemic mice (strain name: B6; 129S7-*Ldlr*^{tm1Her}/J, LDLr$^{-/-}$) (Jackson Laboratory, USA) of 4 weeks old (n=9 per treatment group) were given either 0.026 mg ST, 1 mg (50 mg/kg/day) γT3, δT3, γδT3 or their combinations dissolved in 50 μM DMSO through oral gavage every 24 h. Control group was treated with DMSO. Their body mass was recorded daily. After 4 weeks, the mice were sacrificed for: (a) Lipid profiles. Blood was sampled were done immediately by cardiac puncture after euthanization by carbon dioxide (CO_2) . The serum layer was used for quantitative analyses of total cholesterol, triglycerides and HDL levels, according to the manufacturer's protocol for CHOL, TRIGS and HDL kits (RANDOX Laboratories, UK). (b) Blood biomarker assay. In toxicity observation, serum biomarker measurements of the γ-T3-treated group were compared to the control group. The screened serum biomarkers were albumin (ALB), creatine (Crea), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and alkaline phophatase (ALP). All serum biomarker quantitative analyses were carried out according to the manufacturer's protocol (RANDOX Laboratories). (c) Serum level of γT3 in mice. Forty 5-week-old hypercholesterolemic C57BL/6 mice were given a single-dose intraperitoneal (i.p.) injection of 1 mg (50 mg/kg/day) γT3. Five mice were sacrificed at different time points (10 min, 30 min, 1 h, 3 h, 6 h, 24 h, 48 h and 72 h). Blood samples were collected through cardiac bleeding. To isolate the serum, blood samples were incubated at room temperature for 30 min, followed by centrifugation at 1900 xg, at 4℃ for 30 min. γT3 concentration in serum was analyzed using the HPLC method above. (d) Single acute toxicity test. The maximum tolerated dose (MTD) was determined by increasing doses in different groups of mice until the highest dose without mortality was found. Briefly, 90 hypercholesterolemic C57BL/6 mice (10 in each group) received a single-dose i.p. injection of 1, 2, 4, 8, 12, 16, 20, 30 and 40 mg (50–2,000 mg/kg/day) γT3 in 100 μL injection volume. The weight and survival of mice were observed for 30 days, followed by euthanization by CO_2 inhalation.

Clinical Study on Lipid Profile Improvement by T3

The clinical trial of γδT3 was performed using a double-blind placebo-controlled method. The trial protocol was performed in accordance with the Declaration of Helsinki 1964 (revised in '86/83/89/96/00/02/04), at Takara Clinic (Taisei Bldg, 9F, 2-3-2 Higashigotanda, Shinagawa-ku, Tokyo, Japan). The study protocol was approved by the Tokyo Medical and Dental University Ethics Committee and all patients gave written informed consent. The following ethical considerations were explained to the trial subjects: a) the human rights of subjects who enrolled in this study; b) the possible risks and benefits, adverse events and contribution to the medical field. Consequently, hypercholesterolemic subjects aged 25–55 (mean, 43 years old), with BMI >25 were selected. Exclusion criteria for pooled subjects were usage of drugs or other supplements, food allergy, possibility of pregnancy, or under treatment for chronic or lifestyle diseases. From the 40 initially recruited subjects, the top 50% (20 subjects) with regards to sd-LDL were enrolled in the study. These 20 subjects were randomly separated into 2 groups using StatLight. The T3 group (group A) consisted of 10 subjects (7 male, 3 female). The placebo group (group B) consisted of 9 subjects (6 male, 3 female) following the exclusion of 1 male subject. The final selected subjects had an age range of 49 to 54. During the 8-week treatment, subjects in group A were instructed to consume 4 caps of 30 mg T3 in 270 mg olive oil per day (twice per day, after breakfast and dinner, 2 caps per time). Subjects in group B were given similar instructions to consume 4 caps of 300 mg olive oil per day. Examinations parameters (body weight, height, waist measurement, hip measurement, systolic/diastolic pressures, body fat mass, muscle mass, edema, AST, ALT, ALP, bilirubin, urea nitrogen, creatinine, total cholesterol, triglyceride, HDL, LDL, 4 fractions of lipoprotein, cholesterol: 20 fractions of lipoproteins, triglyceride: 20 fractions of lipoproteins[22]) were taken before and after the supplement intake.

Statistical Analysis

Statistical data wese analyzed using SPSS-Manager. Data are presented as the average ± SD. One-way analysis of variance (ANOVA) was used to compare

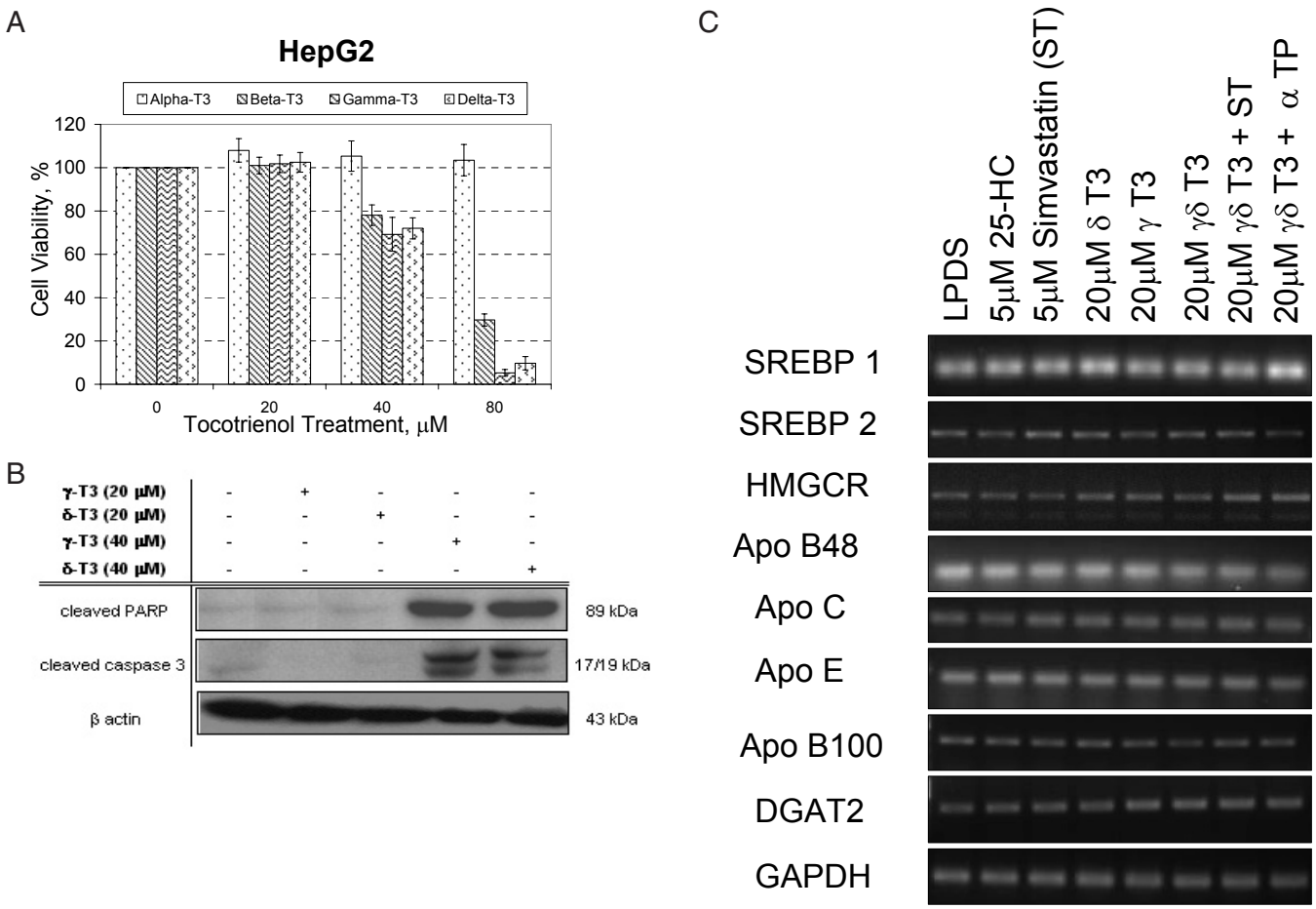

Fig. 1. (A) HepG2 cell viability was examined by standard colorimetric MTT assay. Note that T3 isomers affected the viability of HepG2 cells differently. Except for αT3, the remaining T3 isomers possessed cytotoxic activity at $\geq$40 μM concentration. (B) $\delta\gamma$T3 treatment at 40 μM induced cellular apoptosis, as shown by activation of caspase 3 and poly (ADP-ribose) polymerase (PARP) in HepG2 cells. The $\leq$20 μM treatment concentration was determined to be non-cytotoxic. (C) Treatment with $\delta\gamma$T3, αTP, 25-hydroxycholesterol (25-HC) and simvastatin (ST) did not result in transcriptional changes of cholesterol and fatty acid synthesis genes (SREBP1/2, HMGCR apolipoproteins, DGAT2).

differences between the experimental groups and the control/placebo group. A p value less than 0.05 was considered significant.

Results

Anti-Proliferation Effect of T3 in Human Hepatoma HepG2 Cells

Human hepatoma HepG2 cells were treated with T3 isomers for 24h at increasing dosage (low: 20, μM medium: 40 μM and high: 80 μM). Our results showed that, except for α-T3, the remaining T3 isomers significantly suppressed the proliferation of human hepatoma HepG2 cells at $\geq$20 μM concentration (**Fig. 1A**). The inhibition of cell proliferation was stronger for $\gamma\delta$T3, particularly for γT3, which showed a dose-dependent inhibition. Based on the 50% inhibition concentration, the order of the inhibitory effect was γT3 > δT3 > βT3. Because experiments on lipid-lowering are independent of HepG2 cell proliferation and cell apoptosis (**Fig. 1B**), we used $\leq$20 μM treatment dosage throughout this study.

$\gamma\delta$T3 did not Affect mRNA Expression of Cholesterol and Triglycerides Biosynthesis genes in Human Liver Cell Line HepG2

To study the mechanism responsible for T3-induced lipid-lowering, the transcriptional changes of genes involved in cholesterol[6] and triglyceride[9] biosyntheses with or without $\gamma\delta$T3 treatment were compared by RT-PCR. Treatment of HepG2 cells with LPDS, 25-HC, ST, $\gamma\delta$T3 or their combined treat-

ments did not result in transcriptional changes in SREBP1/2, HMGCR, apolipoprotein B48, apolipoprotein B100, apolipoprotein C, apolipoprotein E, and DGAT2 (**Fig. 1C**). It was worth noting that combined treatment of γδT3 and α-TP did not affect the mRNA of these genes.

γδT3 Downregulated Protein Expression of Cholesterol Biosynthesis genes in Human Hepatoma HepG2

Because SREBP1/2 and HMGCR are key regulators involved in endogeneous cholesterol biosynthesis, the possibility that γδT3-induced lipid-lowering may be attributed to the suppression of these genes was considered. Their protein expression in HepG2 cells treated with γδT3 was measured by Western blotting. As illustrated in **Fig. 2A**, culturing of HepG2 cells in LPDS induced the expressions of all three cholesterol biosynthesis genes, whereas treatment with either FBS or 25-HC repressed their protein expression compared to LPDS. Consistent with previous findings[23], 5 uM ST treatment of HepG2 cells induced HMGCR and SREBP1/2 protein expression. In γδT3-treated HepG2 cells, a dose-dependent decrease in HMGCR protein was observed. which was associated the similar repression of its activators, SREBP1/2 (**Fig. 2B**). These results indicated that γδT3 reduced cholesterol biosynthesis through the suppression of SREBP1/2 and HMGCR. The potency of their suppression was determined to be comparable.

Because αTP was previously reported to attenuate the cholesterol-lowering capability of γδT3[24], we therefore evaluated αTP's ability to block γδT3-dependent HMGCR protein suppression. Our results indicated that 20 μM αTP did not inhibit the effect of γδT3-dependent HMGCR protein suppression (**Fig. 2A**). Protein expression of SREBP1/2 also did not support the role of αTP as an antagonizing factor. In addition, a possible synergistic role between γδT3 and ST was also investigated. Unfortunately, combined γδT3 and ST did not result in enhanced suppression of either HMGCR or SREBP1/2.

γδT3 Upregulated LDL Receptor Expression in Human Liver Cell Line HepG2

LDLr binds to low-density lipoproteins, resulting in the increased clearance of circulating cholesterol. As illustrated in **Fig. 2A**, culturing of HepG2 cells in LPDS induced the protein expression of LDLr, whereas treatment with either FBS or 25-HC repressed it. Consistent with previous findings[25], ST induced the biosynthesis of LDLr to draw cholesterol out of the blood circulation to compensate for the reduced levels of liver cholesterol. More interestingly, our results indicated that γδT3 enhanced the protein expression of LDLr more than ST for the removal of LDL from blood. When γδT3 was combined with αTP or ST, no additional induction of LDLr was observed.

γδT3 Downregulates Protein Expression of Triglyceride Biosynthesis genes in Human Liver Cell Line HepG2

Because suppression of hepatic DGAT2 and APOB100 leads to the reduction of triglyceride synthesis and VLDL secretion from the liver, the possibility that γδT3 induced lipid-lowering attributable to the suppression of these genes was considered. VLDL is the metabolic precursor of LDL and is converted to LDL through the action of lipoprotein lipase, a triacylglycerol lipase that acts upon VLDL while it circulates in the bloodstream[26]. As illustrated in **Fig. 2C**, the protein expression of these two genes was repressed by γδT3 in a dose-dependent manner.

T3 Pharmacokinetics, Single Acute Toxicity and Serum Toxicity Biomarkers After One-Month T3 Supplementation

To evaluate the single acute toxicity of γT3, γT3 was injected intraperitoneally at 9 escalating doses to determine the maximum tolerated dose (MTD), which is defined as the dose at which none of the 10 mice die within a 30-day observation period and at least one of the mice dies at the next highest dose. As shown in **Fig. 3A**, MTD was determined to be 12 mg. For mice receiving 5 i.p. injections per week containing 1 mg γT3 or DMSO blank for 4 weeks, there were no toxicological changes in the serum biomarkers examined (**Fig. 3B**). In addition, the contents of γT3 in vital organs (spleen, heart, lung, kidney, liver) were determined to mainly accumulated in the spleen and liver (**Fig. 3C**).

We also studied the pharmacokinetic behavior of γT3 in plasma after intra-peritoneal administration. Mice were injected with 1 mg γT3 and blood was assayed for γT3 concentration at different time points thereafter. As shown, the plasma γT3 level decreased from 260 ppm to 50 ppm within 30 min after administration (**Fig. 3D**). The level remained stable for at least 72 h.

Lipid Profile of LDLr$^{-/-}$ Hypercholesterolemic Mice After 4-Week γδT3 Supplementation

Mice homozygous for the disrupted LDL receptor allele have an elevated serum cholesterol level of >6.2 mM (or >240 mg/dL) when compared with their wild-type mates fed a normal chow diet (80–100

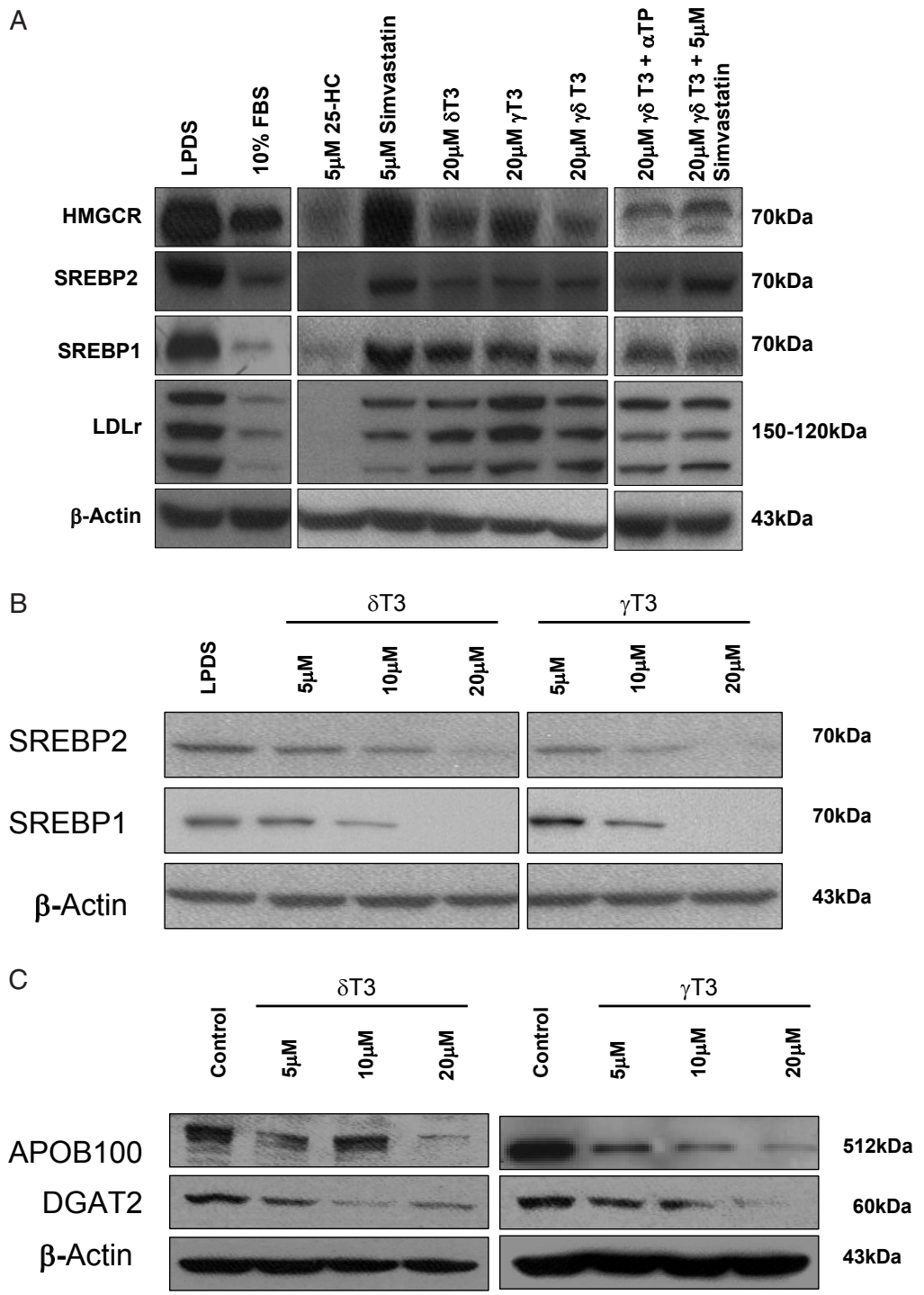

Fig. 2. (A) γδT3 affected fatty acids and cholesterol synthesis by down-regulation of SREBP1, SREBP2 and HMGCR proteins. In constrast, γδT3, in particular γT3, induced a higher LDL receptor (LDLr) expression than simvastatin-treated cells. Lipid-depleted serum (LPDS) acted as a cholesterol-negative control and 10% fetal bovine serum (FBS) acted as a normal cholesterol control, whereas 25-hydroxycholesterol (25-HC) acted as a cholesterol-positive control. (B) γδT3 suppressed SREBP1/2, master regulators of lipid metabolism, in a dose-dependent manner under LPDS co-treatment. γδT3 were found to have comparable inhibitory effect on SREBP1/2 protein expression. (C) γδT3 suppressed DGAT2 and APOB100 protein expression in a dose-dependent manner.

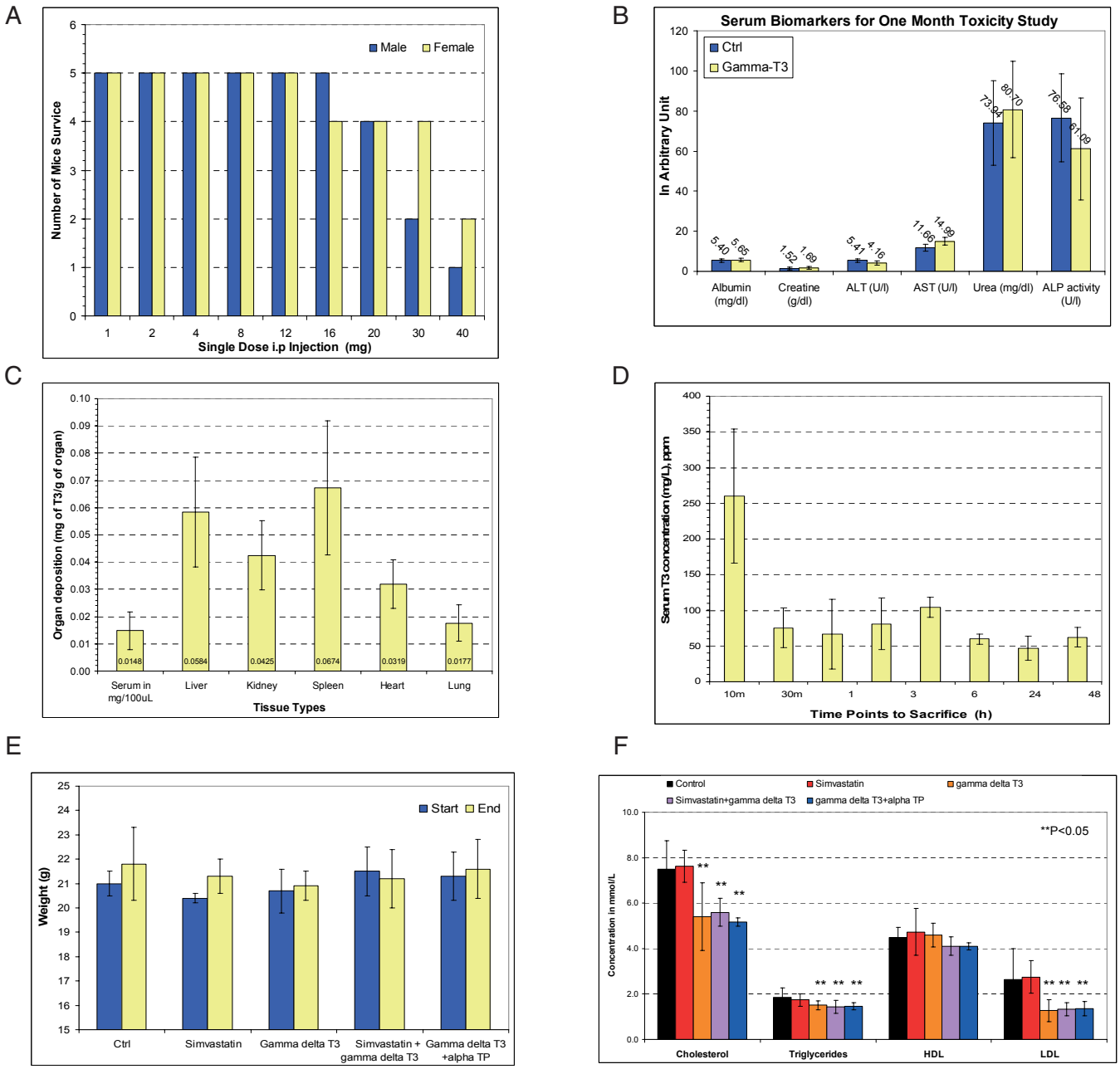

Fig. 3. (A) Ninety LDLr$^{-/-}$ C57BL/6 mice received single-dose 100 μL i.p. injection containing different doses of γT3. The survival of mice was observed for 1 month. The maximum tolerated dose (MTD) was defined as the dose at which none of the 10 mice died within a 30-day observation period and at least one of the mice died with the next highest dose. Based on these criteria, MTD was 12 mg (B) Ten LDLr$^{-/-}$ C57BL/6 mice received 5-dose/week i.p. injection containing 1 mg/day γ T3 or DMSO blank for 4 weeks. No toxicological changes in parameters were observed. (C) γT3 deposition was detected in blood serum and 5 vital organs harvested from γT3-treated LDLr$^{-/-}$ C57BL/6 mice. (D) Forty LDLr$^{-/-}$ C57BL/6 mice received single-dose i.p. injection containing 1 mg γT3. γT3 concentration in serum was analyzed at different time intervals. Plasma γT3 level decreased from 260 ppm to 50 ppm within 30 min after administration. The level remained stable for 72 h. (E) Weight of LDLr$^{-/-}$ C57BL/6 mice before and after $\gamma\delta$T3 treatment remained stable. (F) 1 mg/day $\gamma\delta$T3 administered through oral gavage for 4 weeks reduced the total cholesterol, triglycerides and LDL levels by 25%, 19% and 51%, respectively in LDLr$^{-/-}$ C57BL/6 mice. The HDL level was not affected. It is worth noting that co-administration of 1 mg/day αTP did not attenuate the anti-cholesterolemic property of $\gamma\delta$T3. Also, 0.03 mg/day simvastatin did not result in cholesterol reduction because its activity in transgenic hypercholesterolemic mice is less obvious[28-31]. "**" denotes significant one-way analysis of variance (p value $\leq$0.05) when compared to untreated control group.

Table 1. Characteristics of the hypercholesterolemic human subjects recruited for this study

Characteristics	γδT3 treated group		Placebo group	
	Baseline	End Treatment	Baseline	End Treatment
Subjects				
Male	7		7	
Female	3		3	
Weight (kg)	75.3 ± 11.4	74.4 ± 9.0	77.3 ± 7.4	77.5 ± 7.2
Waist measurement (cm)	92.9 ± 7.3	91.6 ± 6.4	94.6 ± 6.1	95.3 ± 8.0
Hip measurement (cm)	101 ± 7.8	101.3 ± 6.9	102.9 ± 4.4	103.4 ± 5.3
Body fat mass (kg)	21.8 ± 5.3	21.7 ± 4.9	22.6 ± 5.1	23.5 ± 4.9
Body fat percentage (%)	29.1 ± 6.4	29.3 ± 6.8	29.5 ± 7.8	30.7 ± 7.8
Muscle mass (kg)	28.9 ± 6.2	28.3 ± 5.7	29.9 ± 5.8	29.1 ± 5.9
Systolic blood pressure (mmHg)	122.2 ± 10.7	120.3 ± 11.1	133.3 ± 18.4	127.0 ± 13.1
Diastolic blood pressure (mmHg)	80.3 ± 8.6	79.2 ± 6.1	89.4 ± 11.2	85.3 ± 9.8
Triglyceride level (mg/dL)	178.0 ± 77.7	128.4 ± 47.5	129.4 ± 48.4	163.4 ± 102.7
Cholesterol level (mg/dL)	219.0 ± 27.5	218.8 ± 29.8	222.3 ± 13.3	220.8 ± 16.8

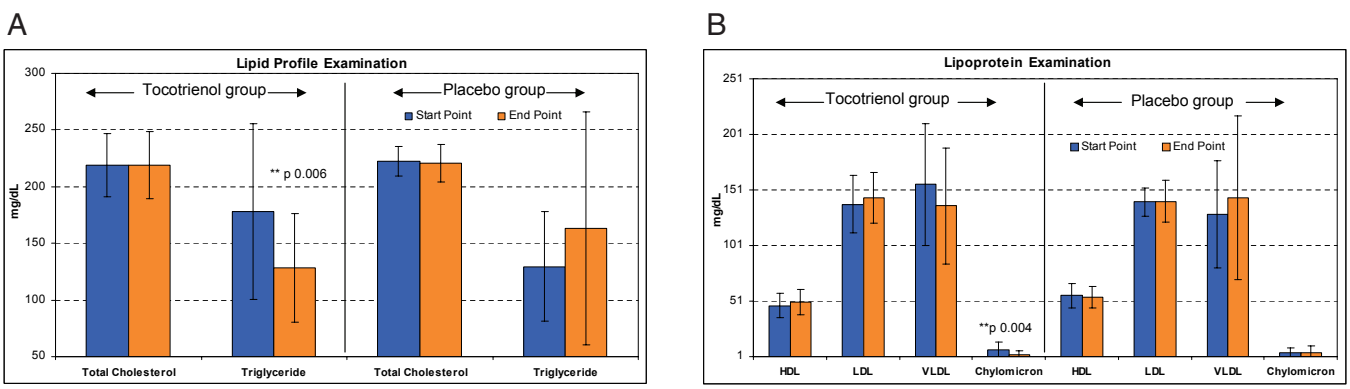

Fig. 4. (A) 120 mg/day δγT3 supplementation for 8 weeks did not affect the total cholesterol, or LDL and HDL levels in hypercholesterolemic patients. It is worth noting that the triglyceride level was reduced in the γδT3-treated group but increased in the placebo group. (B) Fractionation of lipoproteins carrying triglycerides indicated lower triglycerides in the triglyceride-rich lipoproteins VLDL and chylomicron. "**" denotes significant one-way analysis of variance (p value ≤0.05) when compared to level at the start of drug treatment.

mg/dL). Because ST is a first-line lipid-lowering drug in humans and can decrease LDL levels up to 50%, it was given through oral gavage for our *in vivo* study. The experiments were repeated three times.

Throughout the treatment period, mouse weights did not differ among groups (**Fig. 3E**). As illustrated in **Fig. 3F**, serum cholesterol, triglycerides and LDL concentrations fell by 25%, 19% and 51%, respectively, after one-month γδT3 supplementation. The reduction in cholesterol and triglycerides resulting from γδT3 supplementation was more masked than from ST administration. Although ST is effective in humans[27], its activity in hypercholesterolemic mice is less obvious[28-31] as a result of LDLr mutation. It is worth noting that combined supplementation of γδT3 with either αTP or ST did not improve the potency of γδT3.

Lipid Profile of Hypercholesterolemic Patients After 8-Week γδT3 Supplementation

The main characteristics (number of subjects, age, body weight, height, body mass index and initial cholesterol level) of the study population are outlined in **Table 1**. There were no significant changes in these parameters between γδT3-treated and placebo subjects.

To evaluate whether γδT3 supplement reduced serum lipids, the current study evaluated changes in

total serum cholesterol, triglycerides, LDL, HDL lipoproteins and their corresponding subfractions. After 8-week γδT3 treatment, the serum triglyceride level showed a statistically significant reduction (−27.9%, *p* value **0.006), in stark contrast to the placebo group in which the triglyceride level showed an increasing trend (**Fig. 4A**). Consistent with the lower triglyceride level, VLDL and chylomicron level in the treated group were also determined to be lower that with the placebo (**Fig. 4B**). Unfortunately, the serum cholesterol level was not significantly altered in both treated and placebo groups (**Fig. 4A**). Similarly, there were no changes in the 20 fractions of lipoproteins in both treated and placebo groups (**Table 1 Fig. 4** and supplementary data).

Discussion

The present study demonstrated that γδT3 is effective at lowering endogeneous cholesterol and triglyceride biosyntheses in HepG2 cells and hypercholesterolemic mice. Despite positive *in vitro* and *in vivo* results, our 8-week human trial using 120 mg/day γδT3 did not indicate any significant cholesterol reduction at the end of treatment. At the end of γδT3 supplementation, the serum triglyceride level showed statistically significant reduction of 27.9% compared to the baseline (*p* value **0.006). The concentrations of HDL and LDL remained stable during this period.

Lipid-Lowering Biopotency of γδT3 Depends on their Biological Half-Lives

Previous studies using various T3 formulations reached different conclusions regarding the most potent T3 isomer for reducing serum lipids. A double-blind 4-wk study using 200 mg/day γT3 resulted in 31%, 27% and 15% decrease in total cholesterol, LDL, and triglycerides, suggesting that γT3 might be the most effective cholesterol-lowering isomer[12]. Note, however, that these changes in the γT3 group may include a carry-over effect from earlier 4-week T3 supplementation and had no control group. In an other study[32], researchers found that δT3 was equally potent at suppressing the lipid profile. This was substantiated by Western blottings results[13] indicaing comparable suppression of HMGCR by γδT3. Using equimolar treatment dosages, our study consistently determined that the suppression level on SREBP2 was comparable between γδT3 (**Fig. 2B**). Given that γδT3 itself affects the protein abundance of HMGCR through protein regulation and degradation[13], its retention level (biological half-life) in cells is the central feature underlying its biopotency. To this end, it was previously determined that the unsaturated side-chain contributed most to the degradation of T3 to its water-soluble urinary metabolites. In particular, γT3 was metabolized at a rate comparable to δT3[33]. Taken together, it seems likely that γT3 and δT3 possess similar lipid-lowering activity.

Suppression of Triglycerides Biosynthesis by γδT3

To date, few papers published on T3 have discussed its triglyceride-lowering property[32, 34]. The lack of interest in this topic is possibly due to the unclear association between triglycerides and the risk of heart disease. Consequently, the T3 mechanism of action leading to the lowering of serum triglycerides remains unexplored. Recent studies have established that people with elevated triglycerides are indeed at increased cardiovascular risk, strongly associated with other risk factors, including low levels of HDL cholesterol, obesity, insulin resistance, diabetes, and a tendency toward excessive blood clotting[35]. In this study, we determined, for the first time, that T3 inhibited triglyceride biosynthesis through selective downregulation of DGAT2 and APOB100 protein expressions in a dose-dependent fashion (**Fig. 2C**). This direct inhibition of DGAT2 and APOB100 proteins may be a more effective regulation by γδT3 than the signaling cascades for cholesterol biosynthesis genes. As this regulation is independent of sterol-sensing mechanisms, we observed a consistent lowering effect of triglycerides in both hypercholesterolemic mice and humans. Although the number of subjects investigated was insufficient for reasonable assurance of validity, the results were strengthened by the placebo group, which indicated an increasing trend of serum triglyceride level at the end of γδT3 supplementation. The cause of the increasing trend of triglycerides in the placebo group was not completely understood, although the most common metabolic basis seemed to be the altered diet during the trial, which included a holiday period (November 2008−January 2009). Previously, Itoh[36] demonstrated that a high calorie diet composed of fat and simple carbohydrates had significant effects on the serum triglyceride level and body mass index (BMI) in the Japanese population. Consistent with their findings, the average daily calorie intake of subjects in the placebo group during the supplementation period was higher (2,200 kcal/day) than in the tocotrienol group (2,129 kcal/day). Consequently, this led to a concomitant increase in average body fat mass (22.6±5.1 kg to 23.5±4.9 kg), body fat rate (29.5±7.8% to 30.7±7.8 kg) and waist measurement (94.6±6.1 cm to 95.3±8.0 cm) in the placebo group. These parameters were in stark contrast to the tocotri-

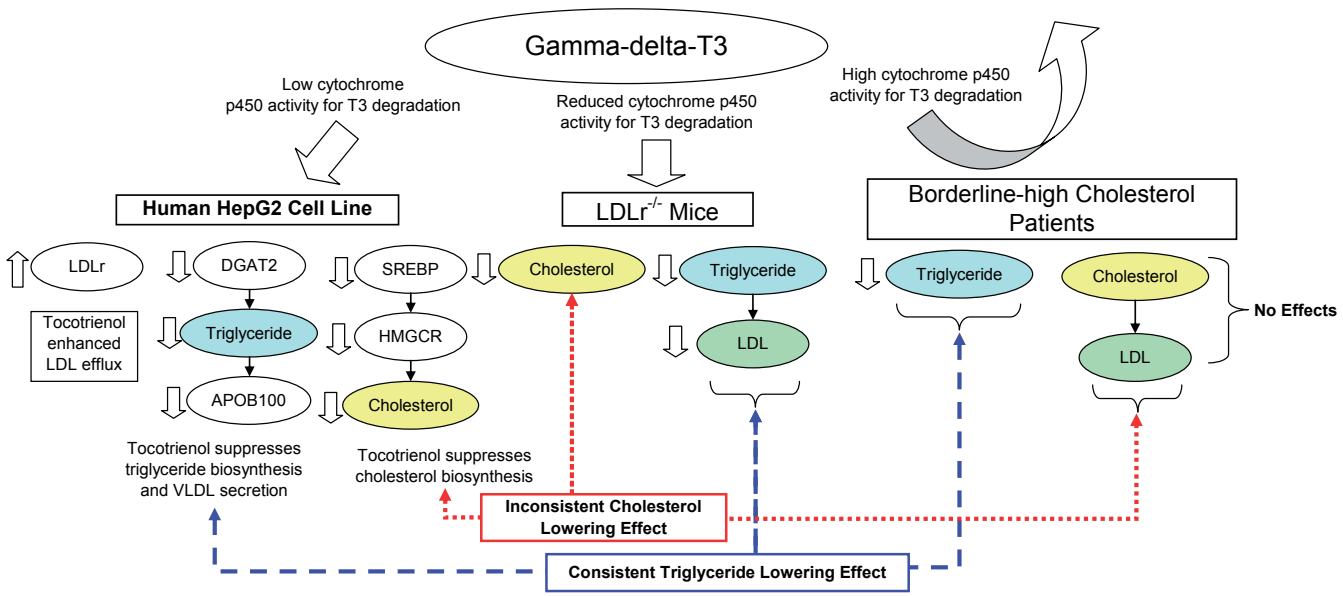

Fig. 5. Summary of this study. Triglyceride reduction may be the primary benefit of ingesting $\delta\gamma$T3.

enol group, which showed a decreasing trend at the end of $\gamma\delta$T3 supplementation (supplementary data). Although these observations remain to be validated in a larger population, they will nevertheless aid in the development of future clinical trials on the triglyceride-lowering effect of $\gamma\delta$T3.

Recently, eicosapentaenoic acid (EPA) purified from n-3 polyunsaturated fatty acids (PUFAs) has shown a proven triglyceride-lowering effect, and was approved by the Ministry of Health, Labour and Welfare of Japan as a treatment for hyperlipidemia and peripheral artery disease based on several large scale trials[37]. Surprisingly, the reduction of the serum triglyceride level in our $\gamma\delta$T3-treatment group (-27.9% at p value **0.006) was greater than that reported for EPA ($<10\%$)[38]. Other triglyceride-reducing agents such as niacin, which prevents the breakdown of fats and decreases VLDL secretion effectively, also lead to observable side effects[39].

$\gamma\delta$T3 Lowered Cholesterol, Triglyceride and LDL Levels in LDLr$^{-/-}$ Mice but not in Humans

We hypothesized that the differential rates of post-absorptive $\gamma\delta$T3 metabolism may be one of the major factors responsible for the discrepancies between *in vitro* and *in vivo* results and our human clinical trial findings. Although HepG2 cells express a variety of liver-specific metabolic functions[40], they are not a suitable cell model to evaluate the lipid-lowering effects of $\gamma\delta$T3 because the genes that encode for cytochrome P450 detoxification activity remain disappointingly low[41], leading to the amplification of $\gamma\delta$T3 effects not reproducible in human liver hepatocytes[42]. In other words, $\gamma\delta$T3 accumulates in HepG2 cells as a result of lower biotransformation potential than primary hepatocytes. In human hepatocytes, $\gamma\delta$T3 is metabolized efficiently by ω-oxidation followed by β-oxidation of the side chain to make a water-soluble substrate for urine elimination[43]. Thus, the lipid-lowering effects observed based on HepG2 cells may not be reproducible in human patients unless a higher dose of $\gamma\delta$T3 is administered.

$\gamma\delta$T3 Lowered LDL Level in LDLr$^{-/-}$ Mice

LDLr plays a critical role in the regulation of plasma LDL levels by mediating approximately two thirds of LDL clearance[44]. Loss of LDLr function leads to decreased LDL catabolism and elevated LDL levels[45]. Our study indicated that $\gamma\delta$T3 lowered serum LDL after 4-week supplementation in LDLD$^{-/-}$ mice. Although the reduction in LDL caused by $\gamma\delta$T3 could be partly accounted for by the suppression of hepatic triglyceride synthesis and VLDL secretion, it could also be due to the ability of apoE/apoB48 particles to bindi to chylomicron remnant receptors for endocytosis. Production of VLDL in mice involves apoB48, and 70% of apoB mRNA in the liver of adult mice encodes the apoB48 isomer[46].

αTP does not Affect the Lipid-Lowering Effects of $\gamma\delta$T3

One published report postulated that the choles-

terol-lowering action of T3 may be attenuated by αTP, as shown in chickens[24]; however, no other researchers have been able to re-produce this finding or the mechanism involved in the attenuation effect. Our *in vitro* and *in vivo* results showed that high-dose αTP (50% αTP) co-treatment with γδT3 did not attenuate the suppression level of HMGCR-related genes and the *in vivo* serum lipid profile. Although a previous study explained that competition exists between T3 and αTP for TP-transport protein (TTP), which primarily binds to αTP, current findings support that oral T3 supplementation is transported to vital organs even in the absence of TTP[47]. There have been reports showing T3 presence in the skin, adipose, heart, lungs, skeletal muscle, brain, spinal cord and liver[47]. In some tissues (e.g. skin tissue serving as a storage organ for vitamin E), the level of T3 exceeded that of TP, indicating the presence of an efficient T3 transport system *in vivo* through blood plasma; therefore, the transportation of T3 may not entirely explain the negating impact of αTP on the T3 lipid-lowering effect. More detailed experiments may be required to elucidate the antagonistic interaction between TP and T3 in lipid-lowering; however, it should be highlighted that αTP did act as an antagonizing agent to T37) when αTP prevented T3 degradation to long-chain carboxychromanols[48]. The latter T3 metabolites serve as anti-inflammation and anticancer agents, and may contribute to the beneficial effects of T3.

Conclusion

The results of the present study demonstrated that the lowering of serum triglycerides may be the primary health benefit caused by ingesting γδT3 in humans (**Fig. 5**). Despite *in vitro* evidence suggesting the suppression of cholesterol pathway genes by γδT3, the absence of cholesterol reduction in our human trial, and others, may have resulted from the high rate of post-absorptive γδT3 degradation in the human liver. Thus, if hepatic γδT3 metabolism can be inhibited, leading to elevated γδT3 concentration, a γδT3 cholesterol-lowering effect can be demonstrated. This hypothesis remains plausible given that sesame seed lignan was previously shown to potently inhibit T3 metabolism (>80%) in hepatocyte cells[33]. Finally, HepG2 liver cells may not be a suitable model to investigate the γδT3 cholesterol-lowering property due to its low cytochrome P450 activities associated with the degradation steps of γδT3.

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Conflicts of Interest

XW Zhang and CP Chang are researchers at South China University of Technology and Duke-NUS Graduate Medical School Singapore, respectively, and have no conflicts of interest. WN Yap, N Zaiden and YL Yap are employees of Davos Life Science, a manufacturer of T3 based in Singapore. S Shunichi is a researcher at Phytopharma based in Yokohama, Japan.

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