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Research paper

PEGylated γ-tocotrienol isomer of vitamin E: Synthesis, characterization, *in vitro* cytotoxicity, and oral bioavailability

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ABSTRACT

Vitamin E refers to a family of eight isomers divided into two subgroups, tocopherols and the therapeutically active tocotrienols (T_3). The PEGylated α-tocopherol isomer of vitamin E (vitamin E TPGS) has been extensively investigated for its solubilizing capacity as a nonionic surfactant in various drug delivery systems. Limited information, however, is available about the PEG conjugates of the tocotrienol isomers of vitamin E. In this study two PEGylated γ-T_3 variants with mPEG molecular weights of 350 (γ-T_3PGS 350) and 1000 (γ-T_3PGS 1000) were synthesized by a two-step reaction procedure and characterized by ^{1}H NMR, HPLC, and mass spectroscopy. The physical properties of their self-assemblies in water were characterized by zeta, CMC, and size analysis. Similar physical properties were found between the PEGylated T_3 and vitamin E TPGS. PEGylated T_3 were also found to retain the *in vitro* cytotoxic activity of the free T_3 against the MCF-7 and the triple-negative MDA-MB-231 breast cancer cells. PEGylated γ-T_3 also increased the oral bioavailability of γ-T_3 by threefolds when compared to the bioavailability of γ-T_3 formulated into a self-emulsified drug delivery system. No significant differences in biological activity were found between the PEG 350 and 100 conjugates. Results from this study suggest that PEGylation of γ-T_3 represents a viable platform for the oral and parenteral delivery of γ-T_3 for potential use in the prevention of breast cancer.

1. Introduction

Vitamin E from palm oil refers to a family of eight related, lipid soluble, compounds that are divided into two subfamilies known as tocopherols (T) and tocotrienols (T_3). Each subgroup has α, β, γ, and δ isomers that differ in the number of methyl substitutions on the chromane moiety and the degree of saturation in their phytyl side chain [1].

Vitamin E was initially discovered as an essential factor for reproduction [2]. The antioxidant properties of this compound were later identified [3]. The poor water solubility of vitamin E, however, limited its clinical use [4]. To overcome this limitation, a PEG conjugate of the α-tocopherol isomer of vitamin E, known as d-alpha tocopheryl polyethylene glycol 1000 succinate or simply vitamin E TPGS was synthesized [5]. This water soluble derivative of vitamin E (1 g/10 mL [6]), was successfully used to treat children with low birthweight, and patients with cholestasis [7]. The tocotrienol isomers of vitamin E were only discovered during the 1960s [8–10]. Remarkably, it was only during the 1990s that the anticancer activities of tocotrienols were identified [11], which established a distinction in the health and therapeutic benefits between the tocotrienol and tocopherol isomers of vitamin E [12].

The γ tocotrienol isomer of vitamin E (γ-T_3) was found to have the most promising anticancer activity [11]. Several studies have reported that γ-T_3 isomer is the most abundant and active as anticancer agent [1,13–22]. Unfortunately, it has also been reported that it is difficult to obtain therapeutic levels of γ-T_3 in the blood and target tissues by simple oral administration due to its poor

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aqueous solubility and limited absorption and oral bioavailability [4,23–26]. To overcome these limitations, our laboratory was engaged over the past decade in developing lipid-based delivery systems for the oral and parenteral administration of γ-T_3 for use in breast cancer prevention and therapy [24–34]. Preliminary results demonstrated that incorporating γ-T_3 into lipid-based delivery systems enhances its oral bioavailability and potentiates its anticancer activity [24,26,32,34,35]. The enhancement in the oral bioavailability of γ-T_3, nonetheless, was limited and was found to be as low as 5.6% at a dose of 1 mg/kg of body weight [25]. In addition to its low aqueous solubility, recent in situ permeability studies have revealed that the intestinal permeability of γ-T_3 is dose dependent when tested at 1, 2.5, 10, 25 and 50 mg/kg dose intervals [4,24–26]. The intestinal absorption of γ -T_3 is a saturable process that was found to be mediated by the Niemann-Pick C1-like 1 (NPC1L1) transporter [24–26]. Moreover, it was found that surfactants (i.e. Cremophor EL (BASF, Mount Olive, NJ) and Labrasol (Gattefossé, Paramus, NJ)) used in lipid-based formulations for the solubilization and oral delivery of γ-T_3 caused a 85% reduction in its cellular uptake due to the surfactant-induced inhibition of the NPC1L1 transporter protein resulting in nonlinear absorption kinetics [25,26]. In addition to passive diffusion and NPC1L1 mediated transport of γ-T_3, vitamin E intestinal absorption was also found to be, at least in part, mediated by the scavenger receptor class B type I (SR-B1) protein [4,23,24].

In light of the aforementioned issues, we aimed to improve the oral bioavailability of γ-T_3 by synthesizing water soluble derivatives. We hypothesized that chemically conjugating γ-T_3 to a polyethylene glycol (PEG) moiety via a succinate linker would enhance the solubility, oral bioavailability, and therapeutic activity of γ-T_3. PEGylation, the process of covalent attachment of PEG, has already been successfully used to enhance the activity of therapeutic proteins and small organic molecules, such as naloxegol (MOVANTIK™), a PEGylated derivative of naloxone, which was recently approved by the FDA [36,37]. While the oral bioavailability and pharmaceutical applications of vitamin E TPGS are well documented [5], the bioavailability and therapeutic activity of the PEG conjugates of the tocotrienol isomers have not been previously reported in the literature. Therefore, the objectives of the current study were to (a) synthesize and characterize two PEGylated γ-T_3 variants with m-PEG molecular weights of approximately 350 (γ-T_3PGS 350) and 1000 (γ-T_3PGS 1000); (b) examine the biological activity of the PEGylated γ-T_3 by evaluating their *in vitro* cytotoxic activity against two human breast cancer cell lines (MCF-7 and MDA-MB-231) and (c) determine the oral bioavailability of the PEGylated γ-T_3 in rats. To the best of our knowledge this marks the first report on the biological evaluation of PEGylated tocotrienol isomers of vitamin E and their self-assembly in aqueous media.

2. Materials and methods

2.1. Materials

Methoxy polyethylene glycols (mPEG 1000 and 350) were provided by INEOS Oxide (Antwerp, Belgium). Tocotrol™ L50P, a tocotrienol rich fraction of palm oil was purchased from Fuji Health Science, Inc. (Burlington, NJ). Triethylamine and succinic anhydride were from Alfa Aesar (Ward Hill, MA). Toluene and Chloroform-d ($CDCL_3$) were from Acros (Bridgewater, NJ). p-Toluenesulfonic acid monohydrate (p-TsOH), hexane, sodium sulfate (Na_2SO_4) and sodium bicarbonate ($NaHCO_3$) were from Avantor (Center Valley, PA). Ethyl acetate was from Pharmco-AAPER (Brookfield, CT). Acetonitrile and dichloromethane were from EMD Millipore (Temecula, CA). Silica gel was from Natland (Morrisville, NC). Pancreatin from porcine pancreas (Lipase not less than 8 USP unites/mg, protease not Less than 100 USP unites/mg, and amylase not less than 100 USP unites/mg) was from Sigma Aldrich (St. Louis, MO). All chemicals and solvents were of reagent grade or higher and were used as supplied without further modification.

2.2. Extraction of γ-Tocotrienol from Tocotrol™ L50P

γ-T_3 was extracted from a 500 g batch of Tocotrol™ L50P, a palm oil fraction that contains approximately 43% tocotrienol isomers of which 40% are γ-T_3. The T_3 rich oil was chromatographed on open column containing 1.5 kg silica gel. Initially the column was flushed with approximately 70 L n-hexane to remove non-vitamin E lipid fractions. The column was then eluted with a gradient solvent system composed of n-hexane and (0–12%) ethyl acetate. Approximately 10–15 L of solvent was used at each ethyl acetate increment. Fractions were collected at different time intervals and analyzed for content by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), mass spectroscopy (MS), and proton nuclear magnetic resonance (^{1}H NMR). Fractions rich in γ-T_3 were concentrated using a Heidolph Laborota 4000 efficient rotary evaporator (Elk Grove Village, IL) to give a yellow to orange viscous oil (~25 g γ-T_3). The content and purity of the γ-T_3 in the product was confirmed by HPLC as previously described [38].

2.3. Synthesis of γ-T_3 succinate

The procedure for the synthesis of γ-T_3 succinate was adapted from Lipshutz et al. [39]. Briefly, triethylamine (0.09 mL) was added to a solution of γ-T3 (1.125 g) and succinic anhydride (0.4 g) in toluene (5.2 mL) and stirred at 85 °C. After 9 h water was added to the reaction mixture, which was then extracted with dichloromethane twice. The combined organic layers were washed with 1 N HCl (3 × 13 mL) and water (2 × 7.5 mL), then dried over anhydrous Na_2SO_4, and finally concentrated in vacuo to yield a yellow liquid. The viscous liquid was further purified by flash column chromatography on silica gel eluted with a 10% ethyl acetate/hexane to 35% ethyl acetate/hexanes gradient to afford γ-T_3 succinate (1 g) as pale, yellow viscous syrup.

2.4. Synthesis of γ-T_3PGS 350 and γ-T_3PGS 1000

Two γ-T_3 PEG variants with m-PEG molecular weights of approximately 350 and 1000 were synthesized via a straightforward two-step route outlined by the scheme in Fig. 1. m-PEG 1000 was selected to be comparable to the commercially available vitamin E TPGS, whereas the shorter m-PEG 350 was chosen to observe the effect of PEG molecular weight on the oral bioavailability and anticancer activity of γ-T_3 PEG. It was reported that the molecular weight of the PEG moiety can affect the availability and absorption of the conjugate molecules when taken orally [37]. A mixture containing γ-T_3 succinate (0.41 g), PEG 350 (0.28 g) or PEG 1000 (0.82 g) and p-TsOH (0.02 g) in toluene (3 mL) was refluxed for 8 h using a Dean–Stark trap. After cooling to room temperature the mixture was poured into a saturated aqueous $NaHCO_3$ solution and extracted with dichloromethane twice. The combined organic layers were washed with saturated $NaHCO_3$ (3 × 7 mL) and brine (2 × 4.5 mL), then dried over anhydrous Na_2SO_4, and finally concentrated in vacuo to afford γ-T_3PGS 350 and γ-T_3PGS 1000 conjugates (0.6 g) as brownish yellow syrups.

2.5. ^{1}H-NMR analysis of γ-T_3PGS 350 and γ-T_3PGS 1000

NMR studies were carried out to confirm the PEGylation of γ-T_3. High-resolution ^{1}H NMR spectra of samples prepared in $CDCL_3$