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Research paper

Synthesis, characterization, and in-vitro antitumor activity of the polyethylene glycol (350 and 1000) succinate derivatives of the tocopherol and tocotrienol isomers of Vitamin E

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ABSTRACT

Vitamin E refers to a group of saturated tocopherol (T) isomers and the biologically more active unsaturated tocotrienol (T_3) isomers. PEGylated α-tocopherol, commercially known as Vitamin E TPGS, has been used as an emulsifier and therapeutic agent for children with vitamin E deficiency. Limited information, however, is available about the PEG conjugates of the tocotrienol isomers of vitamin E. The current work was therefore undertaken to synthesize and characterize the water soluble polyethylene glycol (PEG 350 and 1000) derivatives of T and T_3. Yield and the identity of the synthesized products were confirmed by ^{1}H NMR, mass spectroscopy, HPLC, and thermal analysis. The self-assembly of the PEGylated vitamin E isomers in water at critical micelle concentrations (CMC) was further confirmed by size, zeta, and Cryo-TEM image analysis. While stable at pH 7.4, PEG conjugates were found to rapidly hydrolyze at pH 1.2. Our data showed that PEGylated T_3 isomers were significantly more active as inhibitors for P-glycoprotein than PEGylated T. The in vitro cytotoxicity of the conjugates was also tested against a large panel of normal and tumorigenic cells. Of the conjugates, γ-T_3PGS 1000 and δ-T_3PGS 1000 were found to have the least toxicity against non-tumorigenic breast and pancreatic cell lines, which may be advantageous for its use as functional excipients in drug delivery. The results from the current work have demonstrated the feasibility of synthesizing PEGylated conjugates of vitamin E isomers and highlighted the potential use of these conjugates in drug delivery as functional and safer excipients especially for γ-T_3PGS 1000 and δ-T_3PGS 1000 conjugate.

1. Introduction

Vitamin E represents a family of eight related isomers that are classified into tocopherols and tocotrienols. Each subgroup consists of an α, β, γ and δ isomer that differ in the methyl substitution on the chroman ring and the degree of conjugation in their phytyl side chain (Sylvester et al., 2010). α-Tocopherol, which was the first isomer to be identified in the 1920s was used as an antioxidant (Evans and Bishop, 1922; Evans, 1962). Due to it its poor aqueous solubility, the Eastman chemical company introduced a water soluble derivative in the 1950s by chemically conjugating α-tocopherol with poly ethylene glycol (PEG 1000) using a succinate linker, which became commercially known as "Vitamin E TPGS" or simply TPGS (Guo et al., 2013). TPGS was shown to be effective in reversing or preventing vitamin E deficiency during chronic childhood cholestasis when given orally and was therefore used in the treatment of children with vitamin E deficiency (Sokol et al., 1993). TPGS has also been used as an emulsifier and solubilizer in pharmaceutical products such as Agenerase® (Zhang et al., 2012; Highleyman, 1999) where TPGS increased the solubility of amprenavir in water from 36 μg/mL to 720 μg/mL (Yu et al., 1999). TPGS and the free α-tocopherol isomer were also used to reformulate paclitaxel into an injectable emulsion (TOCOSOL™).

While TPGS has been primarily used as an excipient, it was shown to have an anticancer activity against the MCF-7 and MDA-MB-231 breast cancer cell lines (Neophytou et al., 2014). It was postulated that TPGS can induce apoptosis by inhibiting phospho-AKT and downregulating the anti-apoptotic proteins survivin and Bcl-2 G1/S, and to induce cell cycle arrest by up-regulating P21 and P27Kip1 proteins (Neophytou et al., 2014). TPGS has also been

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shown to inhibit the function of the efflux-pump P-glycoprotein (P-gp), which mediates multi-drug resistance (MDR) to cancer cells by lowering intracellular drug accumulation. TPGS acts on P-gp, in part, by rigidifying lipid bilayers of cell membrane and primarily by inhibiting P-gp ATPase activity (Duhem et al., 2014).

Although extensive research has been reported on the tocopherol isomers of vitamin E including their PEG derivative (TPGS), the tocotrienol isomers (T_3) of vitamin E were only discovered in the 1960s (Whittle et al., 1966; Pennock et al., 1964) and it was not until the 1990s that the anticancer activity of this class of molecules was identified (Pennock et al., 1964). Since then, numerous studies have been reported on the formulation and testing of the tocotrienol isomers against tumor cells in vitro and in animal models (Aggarwal and Nesaretnam, 2012; Sen et al., 2006). Tocotrienols display potent anti-proliferative, apoptotic and autophagic effects against breast cancer cells. The anti-cancer effect of tocotrienols were found to be associated with suppression in growth factor receptor mitogenic signaling pathway and inhibition of epithelial-to-mesenchymal transition in cancer cell lines (Ahmed et al., 2016). The ability of tocotrienols to inhibit the activation and signaling of a wide variety of membrane bound receptors was recently explained (Alawin et al., 2016). It was found that γ-tocotrienol accumulate in and disrupt the integrity of the lipid raft domain within the plasma membrane of breast cancer cells, and this disruption of lipid raft integrity was associated with a reduction in receptor activation and signaling (Ahmed et al., 2016). Based on the plethora of data on the antitumor activity of the free tocotrienol isomers of vitamin E, it was our hypothesis that substituting the α-tocopherol isomer of vitamin E in TPGS with tocotrienols would have a higher pharmacological or antitumor activity, especially against breast and pancreatic cancer, in addition to serving as a solubilizer. Therefore, the overall aim of this study was to compare and contrast between the PEGylated α-tocopherol and PEGylated tocotrienol isomers of vitamin E. More specifically, the objectives of the current study were to (1) design and synthesize PEG conjugates of four vitamin E isomers; α-tocopherol (α-T), α-tocotrienol (α-T_3), γ-tocotrienol (γ-T_3) and δ-tocotrienol (δ-T_3), that have been isolated from Tocotrol™ L50P, a palm oil fraction that contains approximately 43% tocotrienols. Two molecular weight variants of mPEG were used to accomplish this goal; mPEG 350 and mPEG 1000; (2) characterize the PEG conjugates by HPLC, ^{1}H NMR, mass spectroscopy, and thermal analysis; and to analyze the self-assembled micelles of the PEGylated isomers in water for particle size, zeta potential, critical micelle concentration, and by Cryo-TEM microscopy; (3) test the inhibitory effect of the PEGylated vitamin E isomers on P-glycoprotein ATPase activity; and (4) evaluate the in-vitro anticancer activity of the conjugates against the following panel of cell lines: breast cancer (MCF-7 and MDA-MB-231), pancreatic cancer (AsPC-1, BxPC-3, MIA-PaCa-2 and PANC-1), human epithelial mammary gland (hTERT-HME), human pancreatic duct (hTERT-HPNE-1), and the non-tumorigenic human mammary gland (MCF-10). To the best of our knowledge this work, along with our previous study (Abu-Fayyad et al., 2015), mark the first report on the full characterization and in-vitro cytotoxicity evaluation of PEGylated tocopherol and tocotrienol isomers of vitamin E.

2. Materials and methods

2.1. Materials

Vitamin E isomers for the current work were isolated from Tocotrol™ L50P, a viscous tocotrienol-rich fraction of palm fruit oil, that contains approximately 43% tocotrienol isomers (Fuji Health Science Inc., Burlington, NJ). Silica Gel with a 230–400 mesh size, which is suitable for flash (low pressure) chromatography, was from Natland International Corporation (Research Triangle Park, NC). 10 cm d x 60 cm L LG-0000 chromatography column with a fritted disc/PTFE stopcock was custom made by Wilmad-LabGlass Inc. (Vineland, NJ). Ethyl Acetate (EtOAc) from Pharmco-AAPER (Shelbyville, KY). Methoxy polyethylene glycols (mPEG 1000 and 350) were from INEOS Oxide (Antwerp, Belgium). Triethylamine and succinic anhydride were from Alfa Aesar (Ward Hill, MA). Toluene and Chloroform-d ($CDCL_3$) were from Acros (Bridgewater, NJ). Hexanes, AR®, p-Toluenesulfonic acid monohydrate (p-TsOH), sodium sulfate anhydrous (Na_2SO_4) and sodium bicarbonate ($NaHCO_3$) were from Avantor (Center Valley,PA). Acetonitrile and dichloromethane were from EMD Millipore (Temecula, CA). All chemicals and solvents were of reagent grade or higher and were used as supplied without further modification.

2.2. Extraction of vitamin E isomers from Tocotrol™ L50P

The individual tocopherol and tocotrienol isomers of vitamin E were extracted from Tocotrol™ L50P as follows. Approximately 500 gm of Tocotrol™ was first chromatographed on open column containing 1.5 kg silica gel. The column was flushed initially 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 an increasing concentration of ethyl acetate (0–12%). Fractions with pure hexane contained primarily α-T. Increasing the EtOAc to 1% allowed for the elution of pure α-T_3. Increasing EtOAc to 2% allowed for the separation of pure γ-T_3. The δ-T_3 isomer appeared in fractions with >3 and up to 12% EtOAc. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 pre-coated aluminum ALUGRAM® sheets (Macherey-Nagel Inc., Bethlehem, PA). After immersion in samples, sheets were sprayed with 4-anisaldehyde reagent and observed under UV light (254 and 366 nm) using UVGL-15 compact UV lamp (UVP LLC, Upland, CA). Fractions rich in α-T, α-T_3, γ-T_3 and δ-T_3 were concentrated using a Heidolph Laborota 4000 rotary evaporator (Elk Grove Village, IL) to give yellow to orange (α-T) and orange viscous oils (α-T_3, γ-T_3 and δ-T_3). High performance liquid chromatography (HPLC), mass spectroscopy (MS), and proton nuclear magnetic resonance (^{1}H NMR) were performed to confirm the identity of the extracts as discussed in subsequent subsections.

2.3. Synthesis of the succinate derivatives of α-T, α-T_3, γ-T_3 and δ-T_3

The method for the synthesis of α-T succinate, α-T_3 succinate, γ-T_3 succinate and δ-T_3 succinate was adapted from our previous work (Abu-Fayyad et al., 2015). The general reaction scheme is outlined in Fig. 1A. The individual α-T, α-T_3, γ-T_3 and δ-T_3 isomers (1.2 g) were first dissolved in 6 mL toluene. Equimolar amounts of succinic anhydride were then mixed with the isomer solutions. The mixtures were then stirred at 85 °C in a paraffin oil bath. The temperature was maintained using an IKA® RCT heater supported with an IKA® ETS-D4 fuzzy digital thermometer (IKA® works Inc., Wilmington, NC). The reaction was stopped after 9 h and cooled to room temperature. Water was then added and the reaction mixture was extracted with dichloromethane. The upper oily layer was kept and the lower aqueous phase was further extracted with dichloromethane. The combined oily layers were then washed three times with 1 N HCL (7 mL each) and twice with water (8 mL each). Following extraction, the collected oil layers was dried over anhydrous Na_2SO_4, filtered, and concentrated with a rotary evaporator. The concentrate was then mixed with Celite® 545 for further purification on column chromatography. After backing the column with a silica gel slurry (230–400 mesh size), the samples were eluted through the column with the aid of a gradient ethyl acetate/hexane solution with an increasing ethyl acetate