

DMD #31567

**Intestinal Absorption of γ -Tocotrienol is Mediated by NPC1L1: *In Situ* Rat Intestinal
Perfusion Studies**

Bilal Abuasal, Paul W. Sylvester, and Amal Kaddoumi

Department of Basic Pharmaceutical Sciences (B.A., P.W.S. and A.K.), College of Pharmacy,
University of Louisiana at Monroe, Monroe, LA 71201-0470

DMD #31567

Running title:

NPC1L1 mediates γ -tocotrienol intestinal absorption

Corresponding author

Dr. Amal Kaddoumi, College of Pharmacy, Department of Basic Pharmaceutical Sciences,
University of Louisiana at Monroe, 1800 Bienville Dr, Monroe, LA 71201. Email:
kaddoumi@ulm.edu

Number of text pages: 31

Number of tables: 1

Number of figures: 6

Number of references: 40

Number of words in the abstract: 220

Number of words in introduction: 757

Number of words in discussion: 1259

Abbreviations: γ -T3, γ -tocotrienol; γ -CEHC, 2,7,8-trimethyl-2-(β -carboxy-ethyl)-6-hydroxychroman; CYP4F2, cytochrome P450 4F2; Niemann-Pick C1-like 1, NPC1L1; PEG4000, polyethylene glycol 4000.

ABSTRACT

γ -Tocotrienol (γ -T3) is a member of the vitamin E family that displays potent anticancer activity and other therapeutic benefits. The objective of this study was to evaluate γ -T3 intestinal uptake and metabolism using the *in situ* rat intestinal perfusion model. Isolated segments of rat jejunum and ileum were perfused with γ -T3 solution and measurements were made as a function of concentration (5-150 μ M). Intestinal permeability (P_{eff}) and metabolism were studied by measuring total compound disappearance and major metabolite, γ -CEHC, appearance in the intestinal lumen. γ -T3 and metabolite levels were also determined in mesenteric blood. The P_{eff} of γ -T3 was similar in both intestinal segments and significantly decreased at concentrations ≥ 25 μ M in jejunum and ileum ($p < 0.05$), while metabolite formation was minimal and mesenteric blood concentrations of γ -T3 and metabolite remained very low. These results indicate that γ -T3 intestinal uptake is a saturable carrier-mediated process and metabolism is minimal. Results from subsequent *in situ* inhibition studies with ezetimibe, a potent and selective inhibitor of Niemann-Pick C1-like 1 (NPC1L1) transporter, suggested γ -T3 intestinal uptake is mediated by NPC1L1. Comparable findings were obtained when MDCK II cells that express endogenous NPC1L1 were incubated with increasing concentrations of γ -T3, or γ -T3 with increasing concentrations of ezetimibe. The present data show for the first time that γ -T3 intestinal absorption is partly mediated by NPC1L1.

DMD #31567

Introduction

γ -Tocotrienol (γ -T3) is one form of naturally occurring vitamin E present in palm, wheat germ and rice bran (Shookwong et al., 2007; Sundram et al., 2002). Although the vitamin E family of compounds is divided into two subgroups, tocopherols and tocotrienols, all members in both subgroups possess the same general structural features of an aromatic chromanol head and a 16-carbon hydrocarbon tail. However, tocotrienol isoforms have an unsaturated, whereas tocopherols have a saturated phytyl tail (Fig. 1). Recent studies have demonstrated that γ -T3 may provide significant health benefits including anticancer (Shah and Sylvester, 2005) and anticholesterolemic (Song and Debose-Boyd, 2006) activity, as well as acting as a potent antioxidant (Tomeo et al., 1995).

However, studies have also established that it is very difficult to obtain therapeutic levels of γ -T3 in the blood and target tissues by simple oral administration (Sylvester and Shah, 2005). Following its oral administration, γ -T3 is absorbed from the intestine, and transported to the systemic circulation through the lymphatic pathway (Ikeda et al. 1996). The pharmacokinetics of γ -T3 in rat (Yap et al., 2003) and human (Yap et al., 2001) has been reported previously. In humans, while the absolute bioavailability was not determined, γ -T3 relative bioavailability increased 3.5 fold when administered with food (Yap et al., 2001), whereas in rats, γ -T3 oral bioavailability has been found to be as low as 9% (Yap et al., 2003). In addition, in fasting human, plasma tocotrienol concentration was not significantly increased after tocotrienols supplementation (Hayes et al., 1993). γ -T3 is a lipophilic compound with poor solubility (less than 0.01 μ g/ml) and its intestinal absorption increases when taken with food. Food enhances γ -T3 solubility due to the formation of mixed micelles as a result of the stimulation of bile salts and pancreatic enzymes secretions. Furthermore, food increases the lymph lipid precursor pool

DMD #31567

inside the enterocytes that eventually will enhance lymphatic transport (Yap et al., 2001; Kamran et al., 2007). While such increase in γ -T3 solubility when administered with food is significant to enhance its bioavailability, γ -T3 absorption is not complete (Yap et al., 2001; Yap et al., 2003). These findings suggest the existence of other barriers and/or additional mechanisms that are involved in γ -T3 oral absorption and/or transport by primary enterocytes. Tsuzuki et al. (Tsuzuki et al., 2007) investigated the importance of the intestinal uptake properties of γ -T3 on its plasma disposition *in vitro* and *in vivo*. As compared to α -tocopherol, Caco2 cellular uptake and transport of γ -T3 was rapid and consistent with results obtained from *in vivo* studies in mice. However, maximal plasma levels of γ -T3 were much lower than those observed for α -tocopherol (Yap et al., 2003; Tsuzuki et al., 2007).

Oral absorption of drugs can be influenced by specific transporters and metabolizing enzymes present in the endothelial cells lining the gastrointestinal tract. While no previous studies have examined the role of transporters in the intestinal absorption of γ -T3, its metabolism has been investigated. γ -T3 metabolism is catalyzed by the human metabolizing enzyme cytochrome P450 4F2 (CYP4F2) (Sontag and Parker, 2007) into 2,7,8-trimethyl-2-(β -carboxyethyl)-6-hydroxychroman (γ -CEHC) (Swanson et al., 1999). γ -CEHC has been shown to inhibit the production of prostaglandin E2 by inhibiting cyclooxygenase-2 enzyme, which plays a key role in inflammation and its associated diseases (Jiang et al., 2000). CYP4F2 is expressed in human liver, kidney and intestinal cells (Kikuta et al., 1999). When γ -T3 was co-administered to rats with ketoconazole, a CYP4F2 inhibitor (You et al., 2005), urinary excretion of γ -CEHC decreased and γ -T3 concentration in the jejunum increased 3 h following co-administration (Abe et al., 2007). These findings suggest that CYP4F-dependent metabolism of γ -T3 could be a

DMD #31567

critical determinant of its intestinal absorption. However more studies are required to investigate the intestinal contribution to γ -T3 metabolism.

At present, very little is known about γ -T3 absorption by intestinal epithelial cells. The purpose of the current study was to characterize the intestinal uptake and metabolism of γ -T3 utilizing the *in situ* single pass intestinal perfusion model in rats. We investigated the jejunal and ileal permeability and jejunal metabolism of γ -T3. The permeability of γ -T3 was measured at different concentrations ranging from 5-150 μ M, and the intestinal metabolism was estimated by measuring the fraction of γ -T3 metabolized in the intestine. The mesenteric blood levels of γ -T3 and its metabolite, γ -CEHC, were also evaluated. In addition, as γ -T3 intestinal uptake exhibited non-linear kinetics we investigated the possible contribution of the cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) in its transport across the rat intestinal membrane using the *in situ* model, in addition to *in vitro* cell uptake and inhibition studies.

Materials and Methods

Materials and Reagents. γ -T3 was provided by First Tech International Co., Ltd. (Hong Kong). γ -CEHC was kindly supplied by Eisai Co., Ltd. (Tokyo, Japan). Ezetimibe was kindly donated by Schering-Plough Corporation (NJ, USA). Cholesterol and α -tocopherol were obtained from Sigma-Aldrich (St. Louis, MO). Sodium taurocholate and phosphatidylcholine were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). [1,2- 14 C]-PEG4000 (polyethylene glycol 4000, sp. Act.= 0.75 mCi/gm) was purchased from American Raidiolabled Chemicals, Inc. (St. Louis, MO). Unlabeled PEG4000 was purchased from Spectrum Chemical (Gardena, CA). Supplies for cell culture were obtained from American Type Cell Culture Collection (ATCC; Manassas, VA). Other chemicals and reagents were obtained from VWR Scientific (West Chester, PA).

Animals. Male Sprague Dawley rats weighing 260-400 g were acquired from Harlan Laboratories (Houston, TX). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Louisiana at Monroe and all surgical and treatment procedures were consistent with the IACUC policies and procedures. Rats were maintained on a 12 h light/dark cycle before the study and were fasted 12-18 h with water *ad libitum* prior to each experiment.

***In Situ* Rat Intestinal Perfusion Model.** After overnight fasting, rats were anesthetized with intramuscular injection of 50 mg/kg ketamine and 10 mg/kg xylazine mixture, followed by intraperitoneal injection of 40 mg/kg pentobarbital. The small intestine was exposed by midline incision; approximately 15 cm of upper jejunum (proximal to the duodenum) and ileum (caudal to the caecum) were externalized. The segments were then flushed with warm normal saline to remove intestinal contents and cannulated with glass cannulas inserted at the inlet and the outlet

DMD #31567

of each segment and were secured by ligation with silk suture. The inlet tubing of each segment was connected to a 30 ml syringe that was placed in an infusion pump (Harvard apparatus, Holliston, MA). Animals, perfusion solutions, and pump were enclosed in a Plexiglas thermostatically controlled chamber set at 30°C. The perfusate was pumped through the lumen at 0.14 ml/min flow rate. The perfusate solution consisted of γ -T3 prepared as mixed micelles in 1.6 g/l sodium taurocholate and 0.575 g/l phosphatidylcholine in phosphate buffer composed of potassium dihydrogen phosphate 3.9 g/l, and potassium chloride 7.7 g/l. The pH was adjusted to 6.5 with sodium hydroxide.

γ -T3 perfusate solutions were investigated in the concentration range of 5-150 μ M. [14 C]-PEG4000 (0.02 μ Ci/ml) with 0.1% cold PEG4000 was added to the perfusate solution as a marker for water secretion or absorption.

The exposed segments were covered by saline soaked gauze and a plastic film. The first 40 min pre-steady state outlet perfusate were discarded, which represents the stabilization period to reach steady state. Subsequently, the perfusate was collected in vials at 10 min intervals for 100 min. Blood samples, obtained at the end of the perfusion studies from the mesenteric vein, were withdrawn as reported previously (Kaddoumi et al., 2005). Blood samples were centrifuged to separate plasma. Blood collection was followed by rapid removal of the intestinal perfused segments and soaked in ice-cold saline. Plasma and tissues samples were kept frozen at -20°C until analysis. Similar *in situ* jejunal perfusion experiments were conducted with α -tocopherol using 10 and 50 μ M concentrations prepared in mixed micelles as described above for γ -T3.

In experiments other than control perfusate contained, in addition to γ -T3, the NPC1L1 inhibitor ezetimibe at different concentrations were examined.

DMD #31567

Samples Preparation. *γ -T3 sample preparation.* Perfusate samples were diluted with acetonitrile (1:10 or 1:30, depending on the perfusate concentration). γ -T3 was then analyzed by direct injection of 20 μ l onto the HPLC system.

Extraction of γ -T3 from the intestinal tissues was conducted as follows. Collected tissues were allowed to thaw at room temperature and then immediately homogenized (IKA[®] T10 basic Ultra-Turax homogenizer, NC, USA) in normal saline containing 1% ascorbic acid (1:1, tissue weight to saline volume). A 100 μ l of the homogenate was then vortex mixed with acetonitrile (1:6, v/v) for 30s. Samples were then centrifuged (Eppendorf centrifuge 5804R, Hamburg, Germany) at 10,000 g for 10 min. From the collected supernatant 20 μ l were injected onto the HPLC system. γ -T3 extraction from plasma was performed using a modification of the method described by McIntyre et al. (McIntyre et al., 2000). Briefly, to 50 μ l plasma, 100 μ l of 3% sodium lauryl sulfate solution containing 1% ascorbic acid and 125 μ l ethanol were added and mixed. This was followed by the addition of 500 μ l hexane, vortex mixing for 30s, and centrifugation at 10,000 g for 10 min. From the organic layer, 400 μ l were transferred into a vial and evaporated to dryness (Centrivap concentrator, Kansas City, MO). The residues were then reconstituted with the mobile phase, from which 10 μ l were injected onto the LC/MS/MS system.

γ -CEHC sample preparation. γ -CEHC was extracted from the perfusate and plasma samples by liquid-liquid extraction method using ethyl acetate. In brief, to 50 μ l of plasma sample a 1% ascorbic acid (5 μ l) and HCL (12 N, 4 μ l) were added, vortex mixed, and centrifuged for 10 min at 10,000 g. The organic layer was then transferred and evaporated to dryness followed by reconstitution with mobile phase before injection onto the LC/MS/MS system.

DMD #31567

Quantification of γ -T3 and γ -CEHC. Quantification of γ -T3 in the perfusate and tissue homogenate samples was achieved by an isocratic Prominence Shimadzu HPLC system (Columbia, MD). The system consisted of SIL 20-AHT autosampler, SPD-20A UV/VIS detector, and LC-20AB pump connected to a Dgu-20A3 degasser. Data acquisition was achieved by LC Solution software version 1.22 SP1. The chromatographic conditions were Luna 5 μ C18 Column (250 x 4.6 mm id; Phenomenex, Torrance, CA), mobile phase consisted of methanol, ethanol and acetonitrile (40:30:30, v/v/v) delivered at 1.0 ml/min flow rate. The wavelength was set at 210 nm. The total run time was 12 min with retention times of 6.0 and 10.3 min for γ -T3 and α -tocopherol (10 μ M, used as internal standard for γ -T3 experiments), respectively. Standard curves for γ -T3 in the perfusate and tissue homogenate were prepared in the ranges of 0.5-150 μ M and 0.4-195 μ M, respectively. Validation experiments were performed for both perfusate and intestinal tissue homogenates. The method was found to be accurate and precise with inter-day precision of 1.7% for the perfusate, and 13% for the tissue homogenate. The limit of detection of γ -T3 in both matrices was 0.1 μ M.

γ -T3 in plasma, and γ -CEHC in perfusate and plasma samples were analyzed by LC/MS/MS. In brief, the chromatographic separation was performed on a 250 x 4.6 mm Luna 5 μ PFP column (Phenomenex) using Agilent 1100 series LC system (Santa Clara, CA) and 3200 Qtrap LC/MS/MS system (Applied Biosystems, Foster City, CA). The mobile phase used for γ -T3 separation was similar to that used with the HPLC system with the addition of 0.05% acetic acid. While the mobile phase used for the metabolite separation consisted of water and methanol (20:80, v/v) containing 0.05% acetic acid. The analytes were detected by mass spectrometry using electrospray ionization (ESI) interface operated in positive and negative modes for γ -T3 and γ -CEHC, respectively. Instrument control and data acquisition were carried out by the

DMD #31567

Analyst 1.4.1 software (Applied Biosystems/MDS Sciex). The analytes were detected and quantified by MS/MS in multiple-reaction monitoring (MRM) method. The following transitions (precursor > product) were used for quantification; γ -T3: 411>151, and γ -CEHC: 263>219.

Under these chromatographic conditions, the detector signal was linear with respect to γ -T3 concentration over the range 15-366 nM of γ -T3 in plasma. While for the metabolite the calibration curves were linear over the ranges 2-75 nM and 10-75 nM in the perfusate and plasma, respectively. Intraday and interday precision for γ -T3 and γ -CEHC detection in different concentrations for each matrix was evaluated and found to be <16%. MRM chromatograms of γ -T3 (A) and γ -CEHC (B) extracted from rat mesenteric plasma sample obtained from jejunal segment perfused with 50 μ M of γ -T3 are shown in Fig. 2.

Effective Permeability and Fraction Metabolized Determinations. [14 C]-PEG4000 was used as a marker to calculate water flux by comparing the radioactivity counts of [14 C]-PEG4000 in the perfusate before and after the perfusion. No significant difference was found indicating water loss or excretion was minimal, thus no corrections were made for the estimation of analytes concentrations. The P_{eff} of γ -T3 across the rat intestine was calculated based on its loss from the perfusate according to the equation:

$$P_{\text{eff}} = \frac{-Q}{2\pi rL * \ln\left(\frac{C_1}{C_0}\right)}$$

where Q is the perfusate flow rate through the segment (0.14 ml/min), r is the radius of the intestinal lumen (0.2 cm), L is the length of the perfused segment (15 cm), C_0 is γ -T3 concentration at the start of the perfusion (from the entry tubing), and C_1 is the steady state of γ -T3 concentration exiting the perfused intestinal segment.

DMD #31567

The fraction of γ -T3 metabolized (F_{met}) measured in the jejunum was calculated as the concentration ratio of metabolite (γ -CEHC) over total loss of parent γ -T3 from the lumen according to the equation:

$$F_{met} = \frac{(\gamma-CEHC)_{out}}{[(\gamma-T3)_{in} - (\gamma-T3)_{out}]}$$

In the tissue and plasma, γ -T3 and its metabolite concentrations were calculated from their corresponding working calibration curves.

Cell Culture. MDCK II cells were a gift from Dr. R. Govindarajan (University of Georgia, Athens, GA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 2.5% antibiotics (penicillin and streptomycin).

Western Blot Analysis. The analysis of NPC1L1 expression in MDCK II cells was performed as follows: 16 μ g of protein extracts were resolved using 7.5% SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. After blotting, the membrane was blocked using 2% BSA in PBS. The membrane was then immunoblotted with NPC1L1 rabbit polyclonal IgG primary antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and β -actin (C-11) primary antibodies at dilutions 1:200 and 1:3000, respectively and incubated overnight at 4°C. For protein detection, the membrane was subsequently incubated with secondary anti-rabbit IgG antibody for NPC1L1 and anti-goat IgG antibody for β -actin, both labeled with horseradish peroxidase (HRP), at 1:5000 dilution each. The blots were developed using Pierce ECL Western Blotting Substrate Detection Kit (Thermo Scientific, Rockford, IL). Quantitative analysis of the immunoblots was performed using Syngene luminescent image analyzer (Scientific Resources Southwest, Inc., Stafford, TX).

Micellar γ -T3 and Ezetimibe Preparation. The micelles were prepared according to the method described by Narushima et al (Narushima et al., 2008). In brief, cholesterol dissolved in

DMD #31567

ethanol, phosphatidylcholine dissolved in methanol, taurocholate dissolved in ethanol and γ -tocotrienol or ezetimibe dissolved in methanol were mixed and evaporated to dryness under nitrogen. RPMI-1640 serum free medium was then added to prepare the medium for transport experiments. Serial dilutions of the mixed micelles containing ezetimibe were done with ezetimibe-free mixed micelle solution to prepare 0, 2, 20, and 200 μM concentrations of ezetimibe mixed micelles. γ -T3 mixed micelles was prepared at 2 μM . In the inhibition studies, final concentrations of ezetimibe per well were 0, 1, 10, and 100 μM , while for γ -T3 the final concentration was 1 μM per well.

γ -T3 Uptake and Inhibition Studies. Cells were seeded in 48-well plate at a density of 5000 cells/well and cultured for 2 days till cells are confluent. Uptake studies were performed in triplicate. On the day of the experiment, cells were incubated with γ -T3 mixed micelles at rising concentrations (1, 5, 10, 25, and 50 μM) for 60 min. At the end of the incubation period, cells were washed two times with 10 μM taurocholate dissolved in phosphate buffer solution to wash out non specific bound γ -T3. Cells were lysed, and incubated with mixing for 30 min. Aliquot of 100 μl were used for γ -T3 analysis by LC/MS as described above, and 10 μl were used for protein determination. Ezetimibe inhibition studies were conducted in the same manner, except that before the addition of γ -T mixed micelles, cells were pre-incubated with different concentrations of ezetimibe mixed micelles for 30 min followed by the addition of γ -T3 mixed micelles.

Statistical Analysis. Data are presented as mean \pm SEM of at least 3 experiments. Comparison between P_{eff} , F_{met} and tissue-bound γ -T3 and γ -CEHC concentrations were made by one way ANOVA with Tukey's Post-hoc test (JMP[®] 7 software by SAS institute corporation). Differences were considered significant at p value less than 0.05.

Results

Permeability of γ -T3 in Rat Jejunum as a Function of Concentration. To investigate whether γ -T3 exhibits linear or non-linear intestinal absorption kinetics, the effective permeability of γ -T3 was examined by perfusing the concentrations 5, 10, 25, 50, 75, 100, and 150 μ M mixed micelles through the jejunum of the rats. The range of selected γ -T3 perfusate concentrations corresponds to a relevant estimated dose range of 0.5-15 mg in humans. Based on off-setting two orders of magnitude differences in humans and rats in both weight and oral fluid volume of administration, similar drug concentrations in the intestinal lumen of humans and rats would be expected over a given mg/kg dose range. That is, a human dose of 10 mg taken with 8 ounces of water would be estimated to give an initial upper intestinal concentration of 100 μ M.

The P_{eff} results of γ -T3 in the rat jejunum as a function of perfusion concentration are presented in Fig 3. These results show that the permeability of γ -T3 at 10 μ M perfusate concentration ($26.3 \times 10^{-5} \pm 9.2 \times 10^{-5}$ cm/s, n=6) is significantly higher as compared to the other perfusate concentrations ranged from 25-150 μ M ($p < 0.05$). At the lowest perfusion concentration (5 μ M), under the chromatographic conditions used γ -T3 peak was not detected, strongly suggesting that uptake was nearly complete. Nonetheless, when the limit of detection of 0.1 μ M was used to estimate the permeability at 5 μ M perfusate concentration, the results obtained indicated a P_{eff} value of at least 48.5×10^{-5} cm/s (n=4). This value is significantly higher than the γ -T3 P_{eff} at perfusion concentration ranging from 10-150 μ M ($p < 0.05$).

Quantification of γ -T3 and γ -CEHC in Jejunal Tissue, Mesenteric blood, and Perfusate. γ -T3 concentrations analyzed in the jejunal tissue and mesenteric blood over the same range of perfusate concentrations are shown in Table 1. In the tissue, γ -T3 concentrations

DMD #31567

increased with each corresponding increase in perfusate concentration from 10 to 150 μM . At the 5 μM perfusate concentration $\gamma\text{-T3}$ was not detected, but blood $\gamma\text{-T3}$ was shown to be 34.1 ± 3.9 nM. However, mesenteric blood levels of $\gamma\text{-T3}$ over the entire range of perfusate concentrations showed no significant differences ($p>0.08$) between the different treatment groups (Table 1), signifying the role of the lymphatic system in the transport of $\gamma\text{-T3}$ into the blood circulation (Ikeda et al. 1996).

$\gamma\text{-T3}$ is metabolized primarily to free and conjugated $\gamma\text{-CEHC}$ (Freiser et al., 2009). To measure conjugated forms of $\gamma\text{-CEHC}$ in the intestine, acid hydrolysis of the perfusate and blood samples was performed according to the method by Li et al. (Li et al., 2008). Measurements of free and total $\gamma\text{-CEHC}$, in the outlet perfusate and mesenteric blood levels were found to be less or equal to the limit of quantification (≤ 2 nM in the perfusate, and ≤ 10 nM in the mesenteric blood), indicating that the F_{met} of $\gamma\text{-T3}$ was insignificant ($<0.02\%$).

Regional Differences in $\gamma\text{-T3}$ Permeability. The regional differences in $\gamma\text{-T3}$ permeability across the jejunum and ileum were investigated at 2 concentrations (10 and 75 μM) and are shown in Fig 4. Like in the jejunum, permeability of $\gamma\text{-T3}$ at 10 μM was significantly higher as compared to 75 μM ($p<0.05$) in the ileum. In addition, the permeability of $\gamma\text{-T3}$ in the jejunum and ileum at both concentrations were comparable and were not found to be significantly different between the 2 intestinal regions ($p>0.6$).

Jejunal Permeability of $\alpha\text{-Tocopherol}$. Additional studies compared $\alpha\text{-tocopherol}$ and $\gamma\text{-T3}$ P_{eff} values at perfusate doses of 10 and 50 μM . At the 50 μM concentration, the $\alpha\text{-tocopherol}$ P_{eff} value was $11.9 \times 10^{-5} \pm 1.5 \times 10^{-5}$ cm/s ($n=3$) and did not differ significantly from the $\gamma\text{-T3}$ P_{eff} at the same concentration ($9.3 \times 10^{-5} \pm 3.0 \times 10^{-5}$ cm/sec, $n=4$, $p>0.4$). However, at 10 μM concentration the level of $\alpha\text{-tocopherol}$ in the outlet perfusate could not be detected, and the

DMD #31567

permeability was estimated to be $\geq 45.6 \times 10^{-5}$ cm/s (n=3), corresponding to α -tocopherol permeability at concentrations that are lower than its limit of detection (0.25 μ M). At 10 μ M, α -tocopherol P_{eff} was significantly higher ($p < 0.05$) than that of γ -T3 P_{eff} ($26.3 \times 10^{-5} \pm 9.2 \times 10^{-5}$ cm/s, n=6) obtained at the same dose. Furthermore, like γ -T3, α -tocopherol displays significant decreases in permeability when the perfusate dose was raised from 10 to 50 μ M ($p < 0.05$).

***In Situ* Inhibition Studies of γ -T3 Transport with Ezetimibe.** Ezetimibe is a potent and selective inhibitor of cholesterol absorption which works by selective binding and inhibition of NPC1L1 (Weinglass et al., 2008). To investigate the contribution of NPC1L1 to the intestinal uptake of γ -T3, ezetimibe at different concentrations (40, 100 and 200 μ M) was copperfused with γ -T3 at 10 μ M concentration. The results are shown in Fig. 5. At 100 and 200 μ M, ezetimibe significantly reduced γ -T3 P_{eff} from $26.3 \times 10^{-5} \pm 9.2 \times 10^{-5}$ cm/s (in the absence of ezetimibe) to $0.99 \times 10^{-5} \pm 0.19 \times 10^{-5}$ and $2.0 \times 10^{-5} \pm 0.26 \times 10^{-5}$ cm/s, respectively ($p < 0.05$). However, at 40 μ M, while a decrease in γ -T3 permeability was observed ($22.2 \times 10^{-5} \pm 3.0 \times 10^{-5}$ cm/s) this reduction was not significantly different when compared to γ -T3 alone perfusion (Fig. 5). With regard to α -tocopherol, copperfusion with ezetimibe significantly reduced α -tocopherol (10 μ M) effective permeability only at 200 μ M concentration from complete absorption ($P_{\text{eff}} \geq 45.6 \times 10^{-5}$ cm/s) in the absence of ezetimibe to $3.6 \times 10^{-5} \pm 1.6 \times 10^{-5}$ cm/s (ezetimibe 200 μ M, $p < 0.05$).

***In Vitro* Uptake and Inhibition Studies of γ -T3 with Ezetimibe.** In an effort to confirm the existence of carrier-mediated transport, we investigated saturability in MDCK II cells uptake of γ -T3. MDCK II cells have been reported to express endogenous NPC1L1 (Weinglass et al., 2008). Consistent with these studies our Western blotting results demonstrated the expression of NPC1L1 in these cells (Fig. 6 A). The uptake of γ -T3 was determined in the presence of the

DMD #31567

following concentrations: 1, 5, 10, 25, and 50 μM . Figure 6B presents the concentration-dependent cellular uptake of $\gamma\text{-T3}$. The uptake of $\gamma\text{-T3}$ by MDCK II cells was saturable at the concentration range examined and its % uptake was significantly reduced from $73 \pm 5.8\%$ at 1 μM concentration to $17.8 \pm 1.4\%$ at 5 μM ($p < 0.05$, Fig. 6B). To further characterize the NPC1L1-mediated uptake of $\gamma\text{-T3}$, an uptake assay was performed in the presence of ezetimibe. As shown in Fig. 6C, ezetimibe at the concentration range examined (1, 10, 100 μM) significantly inhibited $\gamma\text{-T3}$ uptake by more than 15% when compared to its absence ($p < 0.05$).

DMD #31567

Discussion

The results of this study demonstrate that intestinal uptake of γ -T3 is inversely proportional to the concentration of γ -T3 present in intestinal lumen. Studies showed that the elevation in the concentration of γ -T3 in the intestinal perfusate resulted in a corresponding reduction in the permeability of γ -T3 into the enterocytes. These findings strongly suggest the transport of γ -T3 across the intestinal membrane involved a carrier mediated process. These data also indicate that the carrier mediating intestinal absorption of γ -T3 undergoes saturation when exposure to increasingly higher doses of γ -T3 in the intestinal lumen. This hypothesis would explain why it is difficult to obtain elevated levels of γ -T3 in the blood and target tissue following oral administration, and why increasing the oral dose of γ -T3 does not result in a corresponding increase in γ -T3 bioavailability (Hayes et al., 1993; Yap et al., 2001; Yap et al., 2003).

The oral absorption of drugs is determined by several processes and intestinal permeability is considered one of the major parameters governing this process. Enhanced intestinal absorption displays a direct correlation with increased bioavailability (Lennernas, 2007). In addition, intestinal metabolism plays important role in determining drugs bioavailability (Thelen and Dressman, 2009). While micellar solubilization is important for γ -T3 absorption, it is not the only factor attributed to its absorption into the circulation. Unfortunately, very little is known about intestinal uptake of γ -T3 and it is widely believed that all forms of vitamin E are absorbed by passive diffusion (Kayden and Traber, 1993; Kamran et al., 2007). The aim of the present investigation was to unravel the factors governing the absorption and transport of γ -T3 by the enterocytes.

There are several models that have been used to study the intestinal absorption of drugs. One of the most reliable models is the *in situ* single pass intestinal perfusion in rats (Singhal et

DMD #31567

al., 1998; Kaddoumi et al., 2005). *In situ* intestinal perfusion provides a valuable tool to assess the role of regional differences in the coupled and separate contributions of intestinal drug transport and metabolism to drug absorption variability and dose-dependent pharmacokinetics. Moreover, intestinal permeability determined in rat perfusion studies provides excellent correlations with human absorption data (Amidon et al., 1988; Fagerholm et al., 1996). Using this model and to investigate its uptake by the enterocyte, γ -T3 was prepared as a mixed micelle solution containing, in addition to γ -T3, sodium taurocholate and phosphatidylcholine (Kimura et al., 1985; Eehalt et al., 2004).

To test for the existence of a carrier mediated system for the uptake of γ -T3 in rats, the permeability of γ -T3 as a function of concentration was evaluated. Data from these studies demonstrated that the uptake of γ -T3 by the enterocytes is concentration dependent and involved both saturable and non-saturable processes. Furthermore, regional studies as a function of perfusing concentration did not display regional differences in γ -T3 uptake. In both the jejunal and ileal regions of the small intestine, the P_{eff} of γ -T3 decreased with increased perfusate concentration. While the intestinal permeability exhibited a non-linear uptake behavior, the jejunal tissue concentration was directly related to the perfusate concentration in the range of 10-150 μM . Such consistent pattern between γ -T3 permeability and tissue concentration actually was expected in the range of 25-150 μM , but not at 10 μM . The disappearance of γ -T3 from the lumen, however, is not necessarily to reflect its appearance in the tissue. Several, combined or independent, factors could attribute to this observation at 10 μM including enterocytes metabolism, drug transport at the basolateral side to the mesenteric blood as well as the rate of its lymphatic uptake.

DMD #31567

Although the vitamin E family of compounds is composed of 8 naturally occurring isoforms (α , β , γ and δ tocopherols, and α , β , γ and δ tocotrienols), most available studies have focused on α -tocopherol absorption. *In vitro* analysis of α -tocopherol cellular uptake by Caco2 cells revealed that uptake was concentration dependent (Reboul et al., 2006; Brisson et al., 2008; Narushima et al., 2008). Two carriers have been investigated for their possible role in α -tocopherol transport, NPC1L1 (Narushima et al., 2008) and scavenger receptor class B type 1 (SR-B1; Reboul et al., 2006). Results in the present study showed that the permeability of γ -T3 and α -tocopherol displayed reduced permeability at perfusate concentrations of 50 μ M as compared to 10 μ M, but the permeability of α -tocopherol at 10 μ M concentration was significantly higher than γ -T3. The concentration dependent permeability results of α -tocopherol in the current study is consistent with the *in vitro* studies supporting the participation of a carrier mediated process in its intestinal uptake. Like α -tocopherol, our results support a carrier mediated process associated with γ -T3 intestinal uptake.

Inhibition studies using ezetimibe (inhibitor of NPC1L1) and BLT1 (a chemical inhibitor of SR-B1) provided valuable information regarding the transporter mediated γ -T3 uptake. Initial studies with BLT1 indicated that SR-B1 doesn't play a major role in the transport of γ -T3 (data not shown). On the other hand, inhibition studies with ezetimibe showed that γ -T3 is a substrate for NPC1L1. NPC1L1 is a polytopic protein present on the enterocyte brush border membrane that facilitates cholesterol absorption (Yu, 2008). Ezetimibe is cholesterol lowering agent works by selective and direct inhibition of NPC1L1. Majority of animal, genetic, and biochemical findings support NPC1L1 being the target for ezetimibe (Altman et al., 2004; Davis et al., 2004; Garcia-Calvo et al., 2005).

DMD #31567

Intestinal perfusion of 10 μM of $\gamma\text{-T3}$ with ezetimibe (100 and 200 μM) caused a significant decrease in the permeability of $\gamma\text{-T3}$ suggesting $\gamma\text{-T3}$ as a substrate for NPC1L1. Moreover, this reduction in the permeability of $\gamma\text{-T3}$ caused by ezetimibe was comparable to $\gamma\text{-T3}$ permeability when perfused at high concentrations, i.e. 100 and 150 μM , indicating that NPC1L1 is the primary carrier that contributes to the transport of $\gamma\text{-T3}$ across the intestinal membrane. Compared to $\gamma\text{-T3}$, $\alpha\text{-tocopherol}$ transport was inhibited only at higher concentration of ezetimibe suggesting the possible contribution of another transport system in addition to NPC1L1 (e.g. SR-B1; 35) and/or $\alpha\text{-tocopherol}$ has lower affinity to NPC1L1 when compared to $\gamma\text{-T3}$. Further studies are required to explain this data.

Consistent with the *in situ* intestinal perfusion data, results obtained from the *in vitro* studies using MDCK II cells showed that $\gamma\text{-T3}$ uptake is saturable and its uptake was inhibited by ezetimibe in a dose dependent manner.

NADPH-dependent synthesis of $\gamma\text{-CEHC}$ was demonstrated in human and rat liver microsomes, and functional analysis of several recombinant human liver CYP450 enzymes revealed that $\gamma\text{-T3}$ biotransformation is catalyzed by the enzyme CYP4F (Sontag and Parker, 2002; Tsuzuki et al., 2007). In addition, Abe and colleagues could detect CYP4F mRNA in the rat jejunum and suggested that $\gamma\text{-T3}$ is metabolized to $\gamma\text{-CEHC}$ in the intestine (Abe et al., 2007). However, the present study showed that $\gamma\text{-T3}$ intestinal metabolism to $\gamma\text{-CEHC}$ is minimal and does not contribute to its reported low bioavailability.

In conclusion, in the present study, a saturable transport mechanism of $\gamma\text{-T3}$ on the luminal side of the rat intestine was identified. Using *in situ* rat intestinal perfusion model demonstrated that the intestinal uptake of $\gamma\text{-T3}$ is concentration dependent and a saturable process. Results from the *in situ* and *in vitro* inhibition studies revealed the significant contribution of NPC1L1 to

DMD #31567

the uptake and transport of γ -T3 across the cell membrane. While the current experimental model doesn't investigate whether the permeability reduction would trigger a significant reduction in γ -T3 absorption, revealing and understanding the mechanism of its intestinal uptake is vital to developing delivery systems that are able to improve γ -T3 bioavailability and consequently its therapeutic effect. Further mechanistic studies are currently being conducted in our laboratory to characterize the kinetics of γ -T3 intestinal transport by NPC1L1.

DMD #31567

Acknowledgments

This work was supported by a grant from First Tech International Co., Ltd. (Hong Kong). We thank Dr. Amit Warke for valuable assistance in the *in situ* perfusion studies.

References

- Abe C, Uchida T, Ohta M, Ichikawa T, Yamashita K and Ikeda S (2007) Cytochrome P450-dependent metabolism of vitamin E isoforms is a critical determinant of their tissue concentrations in rats. *Lipids* **42**:637-645.
- Altmann SW, Davis HR Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, Zeng M, Wang L, Murgolo N and Graziano MP (2004) Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* **303**:1201-1204.
- Amidon GL, Sinko P and Fleisher D (1988) Estimating human oral fraction dose absorbed: a correlation using rat intestinal membrane permeability for passive and carrier-mediated compounds. *Pharm Res* **5**:651-654.
- Brisson L, Castan S, Fontbonne H, Nicoletti C, Puigserver A and Ajandouz EH (2008) Alpha-tocopheryl acetate is absorbed and hydrolyzed by Caco-2 cells comparative studies with alpha-tocopherol. *Chem Phys Lipids* **154**:33-37.
- Davis HR Jr, Zhu LJ, Hoos LM, Tetzloff G, Maguire M, Liu J, Yao X, Iyer SP, Lam MH, Lund EG, Detmers PA, Graziano MP and Altmann SW (2004) Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J Biol Chem* **279**:33586-33592.
- Ehehalt R, Jochims C, Lehmann WD, Erben G, Staffer S, Reininger C and Stremmel W (2004) Evidence of luminal phosphatidylcholine secretion in rat ileum. *Biochim Biophys Acta* **1682**:63-71.
- Fagerholm U, Johansson M and Lennernas H (1996) Comparison between permeability coefficients in rat and human jejunum. *Pharm Res* **13**:1336-1342.

DMD #31567

- Freiser H, and Jiang Q (2009) Gamma-tocotrienol and gamma-tocopherol are primarily metabolized to conjugated 2-(beta-carboxyethyl)-6-hydroxy-2,7,8-trimethylchroman and sulfated long-chain carboxychromanols in rats. *J Nutr* **139**:884-889.
- Garcia-Calvo M, Lisnock J, Bull HG, Hawes BE, Burnett DA, Braun MP, Crona JH, Davis HR Jr, Dean DC, Detmers PA, Graziano MP, Hughes M, Macintyre DE, Ogawa A, O'Neill KA, Iyer SP, Shevell DE, Smith MM, Tang YS, Makarewicz AM, Ujjainwalla F, Altmann SW, Chapman KT and Thornberry NA (2005) The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc Natl Acad Sci U S A*. **102**:8132-8137.
- Hayes KC, Pronczuk A and Liang JS (1993) Differences in the plasma transport and tissue concentrations of tocopherols and tocotrienols: observations in humans and hamsters. *Proc Soc Exp Biol Med* **202**:353-359.
- Ikeda I, Imasato Y, Sasaki E and Sugano M (1996) Lymphatic transport of alpha-, gamma- and delta-tocotrienols and alpha-tocopherol in rats. *Int J Vitam Nutr Res* **66**:217-221.
- Jiang Q, Elson-Schwab I, Courtemanche C and Ames BN (2000) Gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proc Natl Acad Sci U S A*. **97**:11494-11499.
- Kaddoumi A, Fleisher D, Heimbach T, Li LY and Cole S (2005) Factors influencing regional differences in intestinal absorption of UK-343,664 in rat: possible role in dose dependent pharmacokinetics. *J Pharm Sci* **95**:435-445.
- Kamran A, Jahangir I and Hussain M (2007) Mechanisms involved in vitamin E transport by primary enterocytes and *in vivo* absorption. *J Lipid Res* **48**:2028-2038.
- Kayden HJ and Traber M (1993) Absorption, Lipoprotein transport and regulations of plasma concentrations of Vitamin E in humans. *J Lipid Res* **34**:343-358.

DMD #31567

- Kikuta Y, Miyauchi Y, Kusunose E and Kusunose M (1999) Expression and molecular cloning of human liver leukotriene B4 omega-hydroxylase (CYP4F2) gene. *DNA Cell Biol* **18**:723-730.
- Kimura T, Nakamura J and Sezaki H (1985) Effect of synthetic surfactants on drug absorption in the presence of a physiologic surfactant, sodium taurocholate, in rats. *J Pharmacobiodyn* **8**:661-668.
- Lennernas H (2007) Intestinal permeability and its relevance for absorption and elimination. *Xenobiotica* **37**:1015-1051.
- Li YJ, Luo SC, Lee YJ, Lin FJ, Cheng CC, Wein YS, Kuo YH and Huang CJ (2008) Isolation and identification of alpha-CEHC sulfate in rat urine and an improved method for the determination of conjugated alpha-CEHC. *J Agric Food Chem* **56**:11105-11113.
- McIntyre BS, Briski KP, Gapor A and Sylvester PW (2000) Antiproliferative and apoptotic effect of tocopherols and tocotrienols on preneoplastic and neoplastic mouse mammary epithelial cells. *Proc Soc Exp Biol Med* **224**:292-301.
- Narushima K, Takada T, Yamanashi Y and Suzuki H (2008) Niemann-Pick C1- Like 1 mediates α -Tocopherol Transport. *Mol Pharmacol* **74**:42-49.
- Reboul E, Klein A, Bietrix F, Gleize B, Malezet-Desmoulins C, Schneider M, Margotat A, Lagrost L, Collet X and Borel P (2006) Scavenger receptor class B type I (SR-BI) is involved in vitamin E transport across the enterocyte. *J Biol Chem* **281**:4739-4745.
- Shah SJ and Sylvester PW (2005) γ -Tocotrienol inhibits neoplastic mammary epithelial cell proliferation by decreasing Akt and nuclear factor kB activity. *Exp Biol Med* **230**:235-241.

DMD #31567

- Singhal D, Ho FH and Anderson BD (1998) Absorption and intestinal metabolism of purine dideoxynucleosides and an adenosine deaminase-activated prodrug of 2',3'-dideoxyinosine in the mesenteric vein cannulated rat ileum. *J Pharm Sci* **87**:569-577.
- Song BL and DeBose-Boyd RA (2006) Insig-dependent ubiquitination and degradation of 3-hydroxy-3-methylglutaryl coenzyme a reductase stimulated by delta- and gamma-tocotrienols. *J Bio Chem* **281**:25054-25061.
- Sontag TJ and Parker RS (2002) Cytochrome P450 ω -hydroxylase pathway of tocopherol catabolism. *J Biol Chem* **277**:25290-25296.
- Sontag TJ and Parker RS (2007) Influence of major structural features of tocopherols and tocotrienols on their omega-oxidation by tocopherol-omega-hydroxylase. *J Lipid Res* **48**:1090-1098.
- Sookwong P, Nakagawa K, Murata K, Kojima Y and Miyazawa T (2007) Quantitation of tocotrienol and tocopherol in various rice brans. *J Agric Food Chem* **55**:461-466.
- Sundram K, Thiagarajan T, Gapor A, and Basiron Y (2002) Palm tocotrienols: New antioxidants for the new millennium. *Inform* **13**:634-640.
- Swanson JE, Ben RN, Burton GW and Parker RS (1999) Urinary excretion of 2,7, 8-trimethyl-2-(beta-carboxyethyl)-6-hydroxychroman is a major route of elimination of gamma-tocopherol in humans. *J Lipid Res* **40**:665-671.
- Sylvester PW and Shah S (2005) Mechanisms mediating the antiproliferative and apoptotic effects of vitamin E in mammary cancer cells. *Front Biosci* **10**:699-709.
- Thelen K and Dressman JB (2009) Cytochrome P450-mediated metabolism in the human gut wall. *J Pharm Pharmacol* **61**:541-558.

DMD #31567

- Tomeo AC, Geller M, Watkins TR, Gapor A and Bierenbaum ML (1995) Antioxidant effects of tocotrienols in patients with hyperlipidemia and carotid stenosis. *Lipids* **30**:1179–1183.
- Tsuzuki W, Yunoki R and Yoshimura H (2007) Intestinal epithelial cells absorb γ -Tocotrienol faster than α -tocopherol. *Lipids* **42**:163-170.
- Weinglass AB, Kohler MG, Nketiah EO, Liu J, Schmalhofer W, Thomas A, Williams B, Beers L, Smith L, Hafey M, Bleasby K, Leone J, Tang YS, Braun M, Ujjainwalla F, McCann ME, Kaczorowski GJ and Garcia ML (2008) Madin-Darby canine kidney II cells: a pharmacologically validated system for NPC1L1-mediated cholesterol uptake. *Mol Pharmacol* **73**:1072-1084.
- Weinglass AB, Kohler M, Schulte U, Liu J, Nketiah EO, Thomas A, Schmalhofer W, Williams B, Bildl W, McMasters DR, Dai K, Beers L, McCann ME, Kaczorowski GJ and Garcia ML (2008) Extracellular loop C of NPC1L1 is important for binding to ezetimibe. *Proc Natl Acad Sci U S A* **105**:11140-11145.
- Yap SP, Yuen KH and Lim AB (2003) Influence of the route of administration on the absorption and disposition of α -, γ - and δ - Tocotrienols in rats. *J Pharm Pharmacol* **55**:53-58.
- Yap SP, Yuen KH and Wong JW (2001) Pharmacokinetics and bioavailability of alpha-, gamma- and delta-tocotrienols under different food status. *J Pharm Pharmacol* **53**:67-71.
- You CS, Sontag TJ, Swanson JE and Parker RS (2005) Long-chain carboxychromanols are the major metabolites of tocopherols and tocotrienols in A549 lung epithelial cells but not HepG2 cells. *J Nutr* **135**: 227-232.
- Yu L (2008) The structure and function of Niemann-Pick C1-like 1 protein. *Curr Opin Lipidol* **19**:263-269.

Legends of Figures

Figure 1. Structures of γ -T3, α -tocopherol, and γ -CEHC.

Figure 2. MRM chromatograms of (A) γ -T3, and (B) γ -CEHC extracted from rat mesenteric plasma sample obtained from jejunal segment perfused with 50 μ M of γ -T3. Eluted fractions from the column containing the compound only were allowed to enter the LC/MS/MS for detection. The peaks correspond to 50.6 and 14.0 nM at 4.21 and 5.39 min for γ -T3 and γ -CEHC, respectively of the same rat.

Figure 3. Effective permeability (P_{eff}) of γ -T3 as a function of luminal concentration obtained from *in situ* perfused rat jejunum, n=3-7. The error bars present the SEM. * $p < 0.05$ compared to permeability from the jejunum at luminal concentrations of $\geq 25 \mu$ M.

Figure 4. Effective permeability (P_{eff}) of γ -T3 obtained from *in situ* perfused rat jejunum and ileum at 10 and 75 μ M luminal concentration. The error bars present the SEM. * $p < 0.05$ compared to the jejunum at 10 μ M; ‡ $p < 0.05$ compared to the ileum at 10 μ M.

Figure 5. Effective permeability (P_{eff}) of γ -T3 (10 μ M) obtained from *in situ* perfused rat jejunum in the absence and presence of the NPC1L1 inhibitor ezetimibe at 40, 100 and 200 μ M. The error bars present the SEM. * $p < 0.05$ compared to γ -T3 permeability at 0 and 40 μ M ezetimibe concentrations.

DMD #31567

Figure 6. A) Western blot analysis of endogenous NPC1L1 in MDCK II cells. Lanes 1-4 represent NPC1L1 analyses from 4 different preparations. B) Effect of increasing concentrations of γ -T3 on its % uptake. The uptake of γ -T3 by MDCK II cells was examined at 37°C for 60 min. The error bars represent the mean \pm S.D. of three determinations. * is significantly different from 1 μ M γ -T3; ‡ is significantly different from 5 μ M γ -T3 ($p < 0.05$). C) Inhibitory effect of ezetimibe on the uptake of 1 μ M γ -T3. The uptake of γ -T3 by MDCK II cells was determined at 37°C for 60 min in medium containing increasing concentration of ezetimibe (1, 10, 100 μ M). The uptake of γ -T3 with increasing concentrations of ezetimibe is presented as % reduction in its uptake relative to 100 % uptake in the absence of ezetimibe. * is significantly different from 0 (no ezetimibe); ‡ is significantly different from 1 μ M ezetimibe ($p < 0.05$).

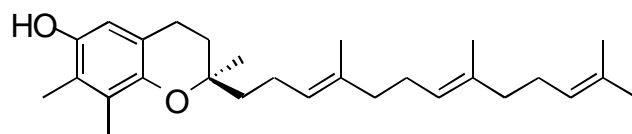
DMD #31567

Table 1. Jejunal tissue (μM) and mesenteric plasma (nM) concentrations of $\gamma\text{-T3}$ at different perfusate concentrations. Values are expressed as Mean \pm SEM.

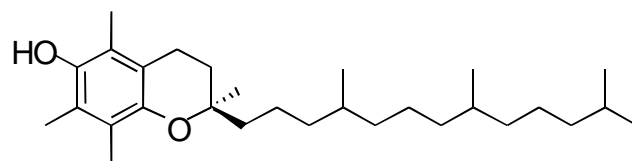
Perfusate Concentration μM (n)	Matrix	
	Jejunal tissue	Mesenteric plasma
5 (4)	BLD [†]	34.0 \pm 3.9
10 (6)	12.2 \pm 2.2	22.1 \pm 10.4
25 (5)	33.9 \pm 6.9	27.1 \pm 11.6
50 (4)	78.0 \pm 3.3	30.4 \pm 10.3
75 (4)	58.5 \pm 8.0	10.3 \pm 2.6
100 (7)	118.4 \pm 12.7	39.8 \pm 16.0
150 (3)	206.2 \pm 53.3	24.4 \pm 9.0

[†] Below limit of detection

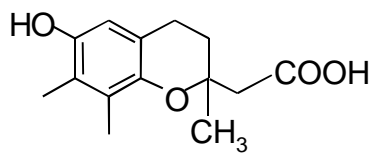
Figure 1



γ -Tocotrienol



α -Tocopherol



γ -CEHC

Figure 2

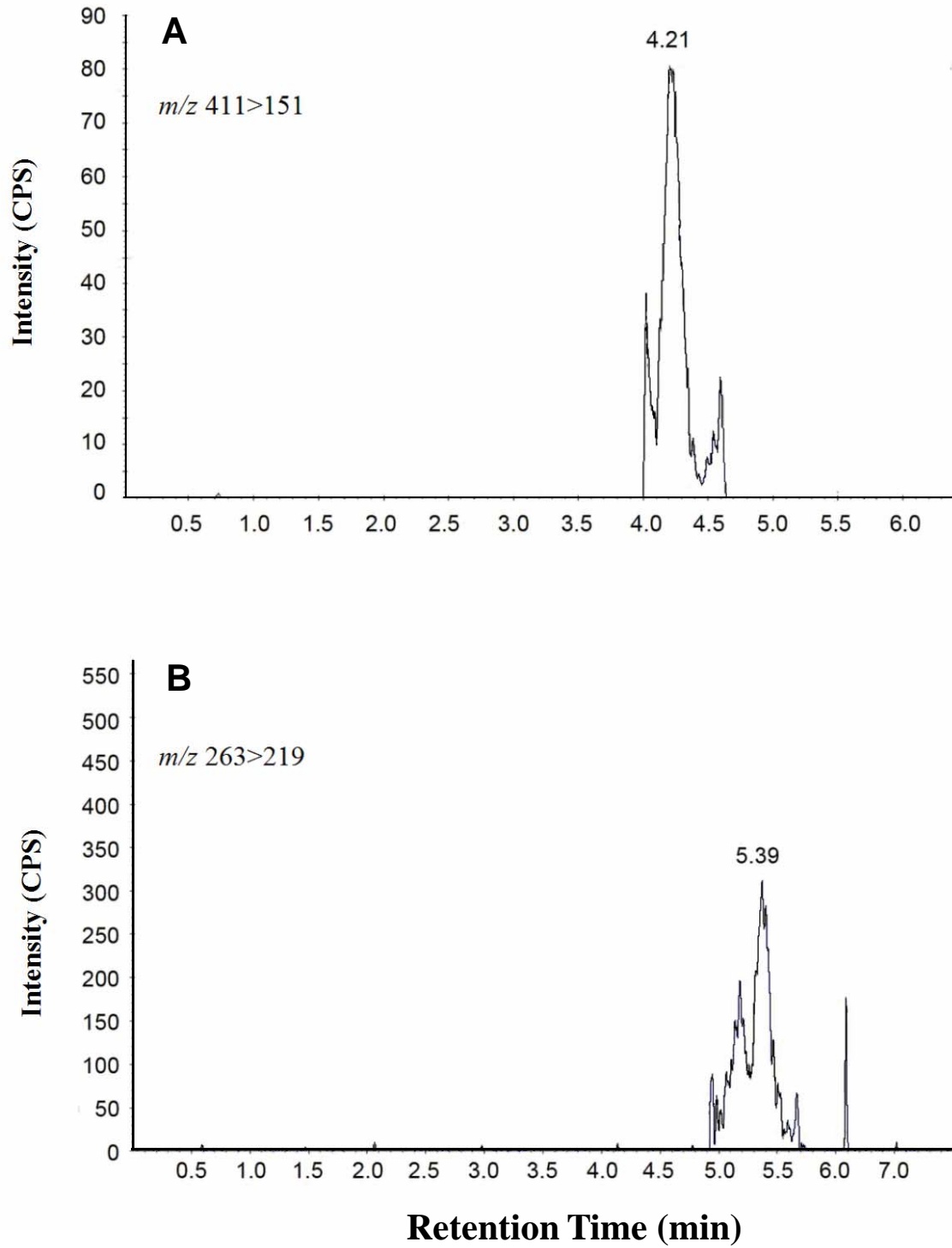


Figure 3

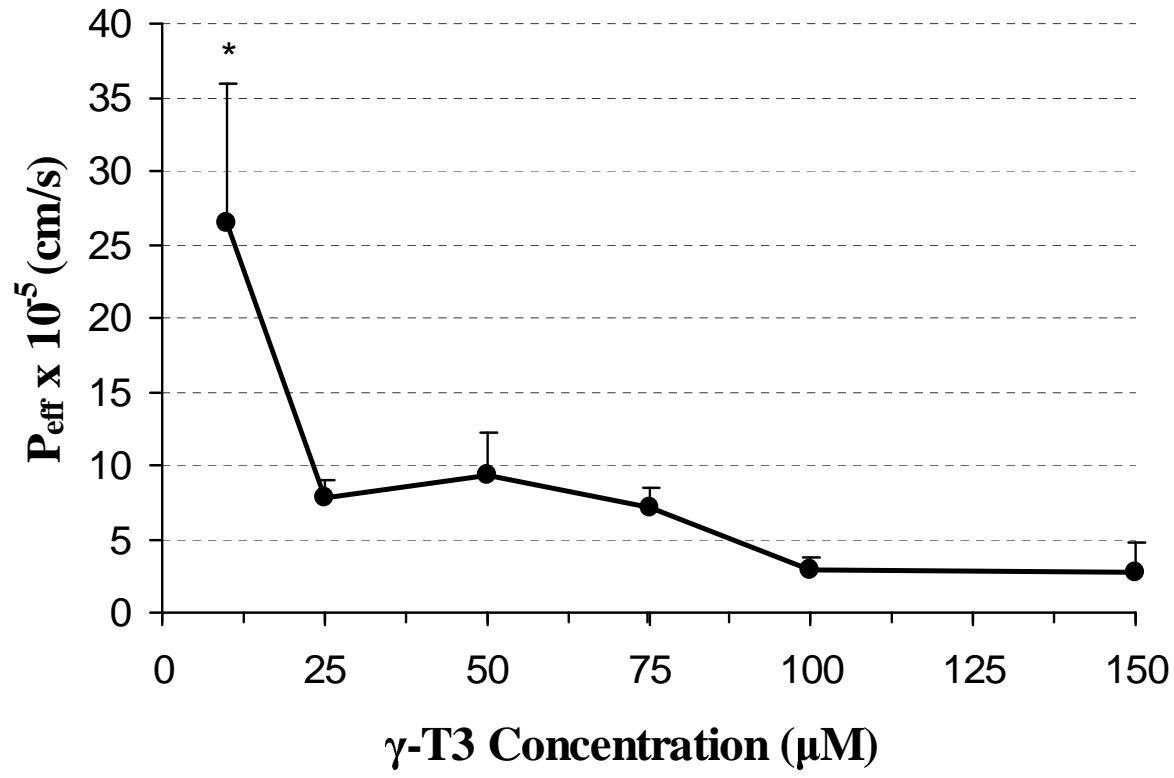


Figure 4

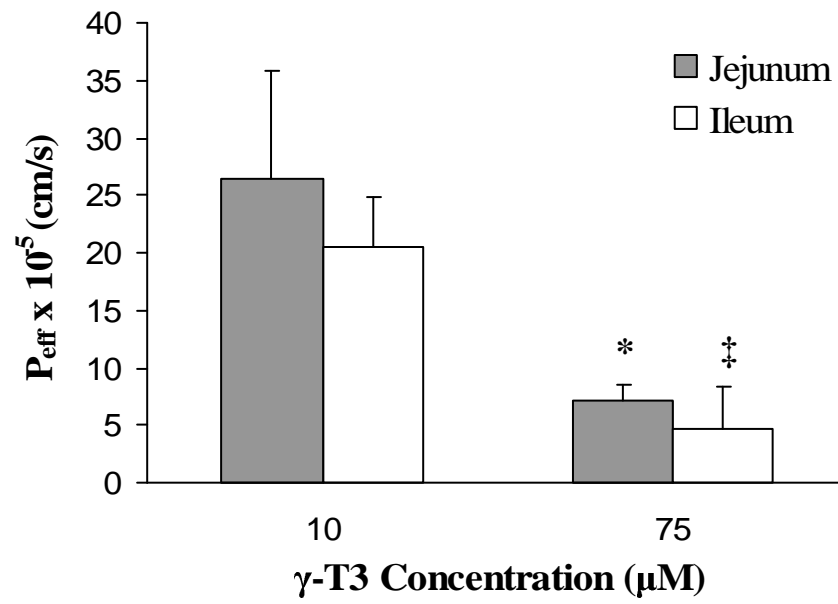


Figure 5

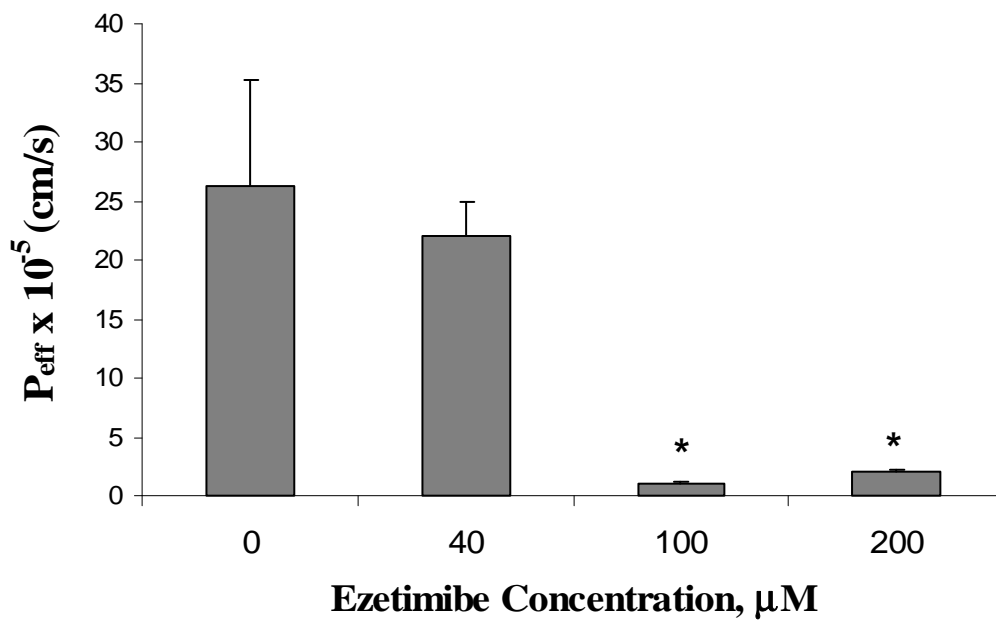


Figure 6

