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Inhibition of cholesterol transport in an intestine cell model by pine-derived phytosterols

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Abstract

We have quantified the inhibition of intestinal cholesterol transport by pine-derived phytosterols using an HT29-MTX intestine cell model that forms a mucus layer similar to that in the intestine. An artificial intestinal fluid consisting of digested fat, bile salt, cholesterol, and phytosterols was formulated in order to mimic the conditions in the intestine. The apparent permeability coefficient \( P_{\text{app}} \) of the positive control, i.e., 0.1 mM of cholesterol solubilized in the artificial intestine fluid, was found to be 0.33 (± 0.17) x 10^{-6} \text{ cm/s}. When 0.1 mM \( \beta \)-sitosterol was solubilized alongside, \( P_{\text{app}} \) was effectively zero, corresponding to a total inhibition of cholesterol transport. A similar strong inhibition was found when commercial pine-derived phytosterols, PinVita™ FSP DuPont, were co-solubilized with cholesterol in the dietary model micelles, leading to \( P_{\text{app}} = 0.06 (± 0.06) \times 10^{-6} \text{ cm/s} \), i.e., 5.5 times lower than the cholesterol positive control. Additionally, the effect of potential oral administration formulations generated by the pine-derived phytosterols was also characterized. The formulations were produced as a liquid formulation to the cholesterol-containing artificial intestine fluid. Six liquid formulations were tested of which four displayed a \( P_{\text{app}} \) in the range of 0 to 0.09 x 10^{-6} cm/s. The other two formulations did not show any inhibition effect on cholesterol transport and even stimulated cholesterol transport. It was furthermore observed that the phytosterols were found in the collected intestine cells but not transported to the basolateral region in the intestinal cell model system.

Key words: HT29-MTX intestinal cell model, apparent permeability coefficient, cholesterol, \( \beta \)-sitosterol, pine-derived phytosterols, sterol transport, PinVita™ FSP DuPont
1. Introduction

Cholesterol is a lipid that plays a pivotal role in many biochemical and biophysical processes. Cholesterol is the precursor of various steroid hormones, bile salts, and vitamin D₃, and is essential for maintaining the proper rigidity of the plasma membrane (Rozner and Garti, 2006). However, excess amount of cholesterol in the blood vessels is the main reason for provoking cardiovascular disease (CVD), coronary heart diseases (CHD) and increased mortality rate (Martin et al., 1986; Weingartner et al., 2011). Cholesterol is supplied to the body through the food (exogenous) or synthesized in liver (endogenous). The exogenous cholesterol is delivered to the apical side of the intestines after food digestion by means of bile salts and lipases. Continuously, the degraded lipid components of the intestines (monoglycerides, fatty acids, and cholesterol) are transformed from hydrophobic material into a colloidal structure, such as micelles, with aid of the bile salts (Carey et al., 1983). Ashworth and Lawrence showed that the 3-25 nm diameter of monoglyceride-oleic acid of digested lipid micelles were optimized to be absorbed into enterocytes (Ashworth and Lawrence, 1966). When the micelles enter into the enterocytes, cholesterol, monoglycerides, and fatty acids are re-esterified as cholesteryl esters, triglycerides, and phospholipids, which are main components of secreted particles (chylomicrons), and then transferred to the basolateral region (Carey et al., 1983; Chang et al., 2009), as illustrated in Fig. 1.

Phytosterols and plant-derived compounds are known for their ability to lower cholesterol levels in the blood stream (Brauner et al., 2012; Jones et al., 1997; Ostlund, 2004; Ostlund et al., 2002; Raederstorff et al., 2003). Phytosterols are lipids with a very similar chemical structure to cholesterol, they are more hydrophobic, and humans cannot synthesize them. Therefore, the phytosterols are available orally from vegetables, plant oils, plant extracts, or plant origin supplements (Ostlund, 2007). The mechanism behind inhibition of cholesterol uptake by phytosterols is still not well characterized. It was previously reported that the phytosterols can inhibit cholesterol uptake through creating bio-unavailable crystals with cholesterol in the stomach and intestines (Christiansen et al., 2003; Rozner and Garti, 2006), limiting the cholesterol solubility in the dietary micelles, blocking metabolic effects of cholesterol in intestinal cells (Brauner et al., 2012; Trautwein et al., 2003). Still, the
effect of phytosterols against cholesterol absorption in the serum appears well established in practice, and consequently phytosterol supplements are used for cholesterol lowering efficacy (Brauner et al., 2012; De Smet et al., 2012). In order to optimize such supplementation there is a need for a deeper and quantitative understanding of the inhibition mechanism.

The intestine cell model system is a very useful tool to investigate drug absorption, nutrient transport, nanoparticle transport, and sterol transport *in vitro* (Ehrhardt and Kim, 2008; Hilgendorf et al., 2000; Langerholc et al., 2011; Rubas et al., 1993; Sun and Pang, 2008). Previously, cholesterol transport was tested in an intestinal model system using Caco-2 cells (Field et al., 1997; Palmgren et al., 2005; Petruzzelli et al., 2009). Caco-2 cells have been widely applied to study drug absorption *in vitro* and to predict drug bioavailability (Rubas et al., 1993; Sun and Pang, 2008). In recent work, HT29-MTX cells were preferred over Caco-2 cell model for studies of lipophilic molecular transport, since the HT29-MTX cell line forms a mucus layer on the surface of the apical region of the cells (Behrens et al., 2001). Because this model system is closer to the natural intestine tissue structure as it consist of mixed population of absorptive cells and mucus-producing cells (Lesuffleur et al., 1990; Lesuffleur et al., 1991). Finally, from the intestine cell model measurements, it is possible to calculate the apparent permeability coefficient (hereafter, $P_{app}$) to be compared with that obtained in the Caco-2 cell model system (Rubas et al., 1993). The $P_{app}$ values of hydrophobic compound transport found when using HT29-MTX cells are lower than the values pertaining to Caco-2 cells due to a physical barrier of the mucus layer formed by HT29-MTX cells (Behrens et al., 2002). $P_{app}$ is the relevant assessment value of drug delivery and compares to the *in vivo* effect indirectly (Rubas et al., 1993).

The aim of the present paper is to quantify the transport of cholesterol in the intestine cell model using a customized artificial intestinal fluid with lipophilic micelles composed of digested fats. Simultaneously, the inhibition of cholesterol transport by phytosterols (β-sitosterol and the commercial pine-derived phytosterol) was characterized by determination of $P_{app}$. In the current study, we have used the non-modified sterols in order not to influence the transport by the chemical modification. Additionally, the commercial pine-derived phytosterol supplement was formulated differently and used to test a potential oral administration.
2. Materials and methods

2.1. HT29- MTX cell culture and trans-epithelial electrical resistance (TEER)

The HT29-MTX cell-line was originally provided by Dr. Thécla Lesuffleur (INSERM UMR S 938, Paris, France). The cell culture medium was high glucose DMEM (Gibco®, Thermo Fisher Scientific, Waltham, MA USA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO USA), 100 unit/ml of Penicillin (Sigma-Aldrich), 100 μg/ml of Streptomycin (Sigma-Aldrich), and 2 mM of GlutaMAX™-I (Gibco®). Cells were grown at 37°C using a 5% CO₂ incubator (NuAire DHD Autoflow, Plymouth MN, USA). The cells were grown in a Petri dish of 10 cm in diameter and in successive subculture until reaching passage 29th. To obtain the cell growth curve, 0.89 x 10⁵ cells/cm² of cells were grown in each wells of a 12-well cell culture plate (Nunc® Sigma-Aldrich) with 3 replications. After an EDTA-trypsin (Sigma-Aldrich) treatment, the number of cells was counted every two days for the growth curve. For the intestine cell model system, the grown cells were transferred into 12-well trans-well culture plates (0.4 μm pore polycarbonate membrane insert, 1.12 cm² culture area, Corning®, Sigma-Aldrich) and seeded in each well using 1 x 10⁵ cells/well (0.89 x 10⁵ cells/cm²). 0.5 ml of medium was added in the donor chamber and 1.5 ml of medium in the acceptor chamber (Fig. 1). The cell culture medium was replaced every day. The trans-epithelial electric resistance (TEER) of grown HT29-MTX cells on the filter membrane in the trans-well plates was measured by a Voltohmmeter (Milipore, Millicell-ERS, Billerica MA, USA) with chopstick-like electrodes. To monitor the variation of TEER, three replicated trans-well plates were separately prepared, and the TEER was measured every second day.

2.2. Two-photon excitation confocal laser microscopy

Cells grown for 15 days on filter membrane were treated with 100 μg/ml of Hoechst 33342 (Invitrogen) to stain the nuclei of the HT29-MTX cells. The stained cells were observed by two-photon confocal laser microscope (LSM 510 ZEISS, Jena, Germany) at 780 nm excitation with a detector of 390-465 nm emission, employing an HFT KP650 filter and an NFT 490 reflection mirror. The three-dimensional view was reconstructed by ImageJ (National Institutes of Health, Bethesda, MD USA).
2.3. Artificial intestinal fluid for cell model system

An artificial intestine fluid (AIF) was prepared with cholesterol, digested fats (monoglycerides and fatty acids), bile salt (sodium taurocholate), and Hanks buffered saline with magnesium and chloride, pH 7.5 (HBSS++, Gibco®). Monoglyceride (MG) (DuPont, Dimondan® MO 90/D, Aarhus, Denmark) and oleic acid (OA) (Sigma-Aldrich) were mixed at a ratio 1:2 at 40°C in a thermomixer (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany). 0.1 mM cholesterol (CORDENPHARMA, Liestal, Switzerland) as positive control, 0.1 mM cholesterol + 0.1 mM of β-sitosterol (Sigma-Aldrich) as co-micelles with pure analytical phytosterol, and 0.1 mM cholesterol + 0.1 mM of β-sitosterol including a commercial Pine-derived phytosterol product (hereafter CPP, PinVita™ FSP, DuPont, which contains 77.6 % β-sitosterol, 11.3% β-sitostanol, 6.6% Campesterol, 1.2% Campesterol, 0.7% Stigmasterol, and over 3% of other sterols) as co-micelles with natural phytosterol extract, were dissolved with 0.5 mM of MG, 1 mM of OA, 33 mM of sodium taurocholate (Sigma-Aldrich) in HBSS++. The three negative controls were prepared as HBSS++ (Neg. Cont I), HBSS++ with 33 mM of sodium taurocholate (Neg. Cont II), and HBSS++ with 33 mM of sodium taurocholate and 1:2 molar ratio of MG:OA mixture (Neg. Cont III). In details, 1 mM of cholesterol, 1 mM of cholesterol + 1 mM of β-sitosterol, and 1 mM of cholesterol + 1 mM β-sitosterol in CPP (calculated from 77.6% of β-sitosterol) in each of 50.7 μl of the 1:2 molar ratio of MG:OA mixture (hereafter, MG/OA) were prepared and then, the each of 2.54 μl of dissolved 1 mM cholesterol, 1 mM cholesterol +1 mM β-sitosterol, and 1 mM cholesterol+1 mM CPP in MG/OA was added in drop wise in 5 ml of 33 mM sodium taurocholate in HBSS++ and 2.54 μl of the only MG/OA was added for the Neg. Cont III. The solutions were separately in the glass vials and heating up to 40°C, stirring overnight on a thermo stirrer (IKA® RT10P, London, UK). The fat mecelles were produced in 33 mM sodium taurocholate in HBSS++. The CPP was tested in two ways; co-micelle solubilization with cholesterol in MG/OA in the process described above as well as mimicking of potential oral administration.

When mimicking the oral administration, the liquid CPP of six different formulations followed a pH change to mimic the conditions of acidic stomach and neutral intestine. Initially the liquid formulations were stirred overnight, followed by a decrease in pH to 2 using HCl. The low pH formulations were stirred for 1 hour.
Subsequently the formulations were neutralized to pH 7 using NaOH and finally tested in the cell model. The pH change was measured by a pH meter (EUTECH instrument pH510, Landsmeer, Netherland) at room temperature. In order to ensure precise β-sitosterol concentration in CPP, care was taken to adjust this to identical concentrations of 0.1 mM by volume adjustments. The CPP formulations have different concentration of β-sitosterol and the used volume was hence recalculated each to reach a concentration of 0.1 mM β-sitosterol. The pH-adjusted solutions were added drop wise in 5 ml of 0.1 mM of cholesterol-MG/OA micelles and 33 mM sodium taurocholate in HBSS++, i.e., the same composition of the positive control.

The particle size and the zeta-potential were measured by dynamic light scattering (DelsaMax pro, Beckman-Coulter Indianapolis, USA). All particles were measured at 20°C in triplicates. The cell model system was implemented as follows: 15 days of cultured cells were prepared in 12-well trans-well plates, 0.5 ml of the prepared fluidic samples (Table 1) were added in the donor chambers and 1.5 ml of HBSS++ were placed in the acceptor chambers after washing the cultured cells three times with HBSS++. Each sample was prepared in three replicas. The treated cells were incubated on the shaking incubator at 37°C for 1 hour with mild shaking (70 rpm) in a shaking incubator (ES-20 BIOSAN, Riga, Latvia). After incubation, 0.5 ml of solution was collected from both the donor chambers and the acceptor chambers. Additionally, the HT29-MTX cells were washed with HBSS++ three times and collected by cell scrapers (24 cm, TPP® Trasadingen, Switzerland) with 0.5 ml HBSS++ for HPLC sterol analysis in the intestinal cells.

2.4. Atomic force microscopy

Atomic force microscopy (AFM) was used to detect and visualize input micelles originating from the donor chamber of the positive control, and following the incubation with the reconstructed AIF, in order to monitor the transported micelles from the acceptor chamber. Since the cholesterol-MG/OA micelles showed a negative zeta-potential (Table 1), the silicon wafers, used to adhere the micelles, were modified by 3-aminopropyl triethoxysilane (APTES) (Sigma-Aldrich) to achieve a positively charged substrate that could attract the negatively charged micelles. The ~1 cm² cut silicon wafers (Si (100), Plano GmbH, Wetzlar, Germany) were cleaned with a boiling solution of 4:1:1 H₂O (Ultrapure water from a integral 5 unit, Millipore
A/S, Billerica, MA USA), NH₄OH (28-30%, Sigma-Aldrich), and H₂O₂ (34.5-36.5%, Sigma-Aldrich) for 10 minutes and subsequently rinsed with ultrapure water, followed by cleaning in a plasma cleaner for 10 minutes (Harrick Plasma, Ithaca NY, USA). The clean wafers were hydrolyzed with 5% APTES in 95% ethanol (Kemetyl, Køge, Denmark) at pH 4.5-5.5 for 2 hours on a rack platform. The excess amount of APTES solution was removed by washing with 95% ethanol 3 times. The organosilane-modified substrates were cured at 100°C overnight and stored in vacuum. The micelle solutions were mixed in 1:1 ratio with a 2% osmium tetroxide (Sigma-Aldrich) solution, spread on the organosilane-modified silicon wafers, and incubated for 1 hour. The wafers were then washed with ultrapure water and dried under nitrogen gas. The prepared silicon wafers with micelles were visualized by AFM (Nano Wizard I, JPK Instruments AG, Berlin, Germany) in intermittent contact mode (tapping mode). Cantilevers had a tip radius lower than ~7 nm and a force constant of ~40 N/m (PPP-NCHR, Nanosensor, Neuchatel, Switzerland) operated at ~330 kHz. Visualization and subsequent image analysis were accomplished using SPIP™ (Scanning Probe Image Process, Image Metrology A/S, Hørsholm, Denmark).

2.5. Sterol quantification by high performance liquid chromatography (HPLC)

The total amounts of sterols, including free and esterified sterol, were quantified from the 0.5 ml solution of the donor chambers and acceptor chambers as well as the scraped cells in 0.5 ml of HBSS++ buffer. The samples were hydrolyzed by mixing 0.5mL of sample with 0.5mL of 5% KOH in methanol followed by shaking at 60°C for 20 min (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany). Sterols were extracted from the reaction mixture by the use of supported-liquid extraction in a Biotage ISOLUTE SLE+ tube (Biotage, Uppsala, Sweden) with a sample capacity of 1ml. Samples were eluted with 2 times of 1.5ml of hexane. The combined extracts were evaporated under at stream of nitrogen at 60°C in a block heater (Stuart SBH200D/3 equipped with a SBHCONC/1 sample concentrator, Bibby Scientific, Stone, UK). Samples were then dissolved in 150 µl of methanol/acetonitrile,30/70, and filtered with 0.2 µm PTFE syringe filters (VWR, Søborg, Denmark). The sterols were then quantified by HPLC on a Shimadzu Prominence HPLC consisting of a DGU20-A5 in-line degasser, LC-20AD pumps, SIL20A-HT autosampler, CTO 10 Column oven and a SPD-20A UV detector (Shimadzu, Kyoto, Japan). Samples were separated
on a Supleco Ascentis® Express RP-Amide 15 cm x 2.1 mm, 2.7 µm column (Sigma-Aldrich) with a mobile phase of acetonitrile/methanol, 90/10, flowing at a rate of 0.3 ml/min. The sterols were detected in the UV-detector at 210 nm and quantified by standards of the pure compounds, cholesterol and β-sitosterol.

2.6. Calculation of sterol quantification

The concentration of cholesterol and β-sitosterol in the donor chamber, intestine cells, and acceptor chamber was measured by HPLC. The amount of transported cholesterol was compared with negative controls and the apparent permeability coefficient \( P_{app} \) was calculated by the following equation (Hilgendorf et al., 2000).

\[
P_{app} = \frac{dQ}{dt} \frac{V_a}{A C_0}
\]

where \( dQ/dt \) is the steady-state flux of sterols across the HT29-MTX cell layer calculated after 1 hour incubation (mM/s), \( V_a \) is the volume of acceptor chamber, \( A \) is the surface area of absorbing membrane in the trans-well plate (1.12 cm\(^2\)), and \( C_0 \) is the initial concentration of cholesterol in donor chamber (0.1 mM) (Hilgendorf et al., 2000). In order to calculate the cell permeability coefficient \( P_c \) for cell absorption of sterols, we calculated the flux defined as the mass of substrate through a given cross-sectional area in a given time. \( P_c \) can be calculated from the flux and the initial concentration of substrate as follows (Steffansen et al., 2010).

\[
J = \frac{m}{A t}
\]

\[
J = P_c C_0
\]

\[
P_c = \frac{J}{C_0}
\]

where \( J \) is the flux, \( m \) is the mass of transported sterols determined by the HPLC values from collected HT29-MTX cells, \( A \) (cm\(^2\)) is the cross-sectional area (1.12 cm\(^2\)), \( t \) is incubation time (s), and \( C_0 \) is the initial concentration in donor chamber (µg/ml).
3. Results

3.1. HT29-MTX cell growth and trans-epithelial electrical resistance

HT29-MTX cells were seeded at a concentration of $0.8 \times 10^5$ cells/cm$^2$ and reached confluence in 10 days, with a concentration of $4 \times 10^5$ cells/cm$^2$ (Fig. 2A). The cells were then grown continuously to an almost three times higher number of cells, compared with the confluence, in 15 days and showed stationary state from then on. After reaching the stationary state, the HT29-MTX cells obtained a tissue-like property and we observed a fluctuation in the growth curve and a high value of standard error after 15 days of culture. The trans-epithelial electrical resistance (TEER) showed a concomitant increase in resistance that accompanied the growth of cells to confluence and did not reach its maximum value until at 15 days in culture but decreased after then (Fig. 2B). Similarly to the growth curve, the TEER curve was fluctuating after 15 days of culture (Fig. 2B). Thus, 15 days in culture was the optimal culture time for trans-epithelial experiments.

3.2. Multilayered structure formation of HT29-MTX cells

As shown in Fig. 3, the HT29-MTX cells that were grown for 15 days on the trans-well plate displayed a folded shape and were multilayered when viewed under the confocal laser microscope. The nuclei of the cells were stained with Hoechst 33342 and can be seen in the z-axis stacked images obtained by two-photon confocal laser microscopy. The XZ axis and YZ axis images showed a clear multilayered structure (Fig. 3A). The three-dimensional view of the reconstructed image by ImageJ showed the folded shape of the intestine tissue-like structure (Fig. 3B).

3.3. Physico-chemical properties of artificial intestine fluid

Artificial intestine fluid (AIF) was defined as the input solution in the donor chamber for cholesterol transport. Three negative controls were used in the study and prepared to assess the intestinal cell model reaction to the buffer, bile salt, and monoglyceride/oleic acid without cholesterol. Table 1 shows the composition of the tested AIF and the physico-chemical properties of micelles of fats (monoglycerides, oleic acids, cholesterol, and phytosterols). Using dynamic light scattering, no
particulate matter were detected in the buffer control (negative control I). From the bile salt control (negative control II) to the sterol-included AIF had very small particles, from 2.3 nm to 3.1 nm in diameter. In addition, the zeta-potential of the particles was negative, -24 mV and -14 mV in negative control II and negative control III, respectively. When sterols were added, the zeta-potential dropped from -44.1 mV to -51.2 mV. The particle sizes for the potential oral administration test of the cholesterol-micelles and the liquid formulations of the commercial pine-derived phytosterol varied from 23.4 nm to 590.6 nm in diameter and the zeta-potential of the formulation-included particles in AIFs displayed negative values in the range of -45 mV to -55 mV (see Table in Ref [1])

3.4. Trans-Epithelial Electrical Resistance (TEER) of HT29-MTX upon incubation with artificial intestine fluid

As shown in Table 2, incubation of the cell culture with all of AIFs increased the trans-epithelial electrical resistance (TEER) of HT29-MTX within 1 hour by anywhere between 12 – 65 Ω·cm². Especially, cholesterol-containing AIF (positive control) showed about 20% higher TEER value than negative controls (Table 2). A similar behavior was also observed for the TEER of the formulations for the potential oral administration of the commercial pine-derived phytosterol (CPP), which all samples contained cholesterol (also listed in Table 2). Those cholesterol-containing AIF plus formulations caused an increase the TEER value of 21 Ω·cm² in average, with the exception of formulation D with an increase of 71 Ω·cm² that is considered as an outlier. Interestingly, the micelle formation of cholesterol together with β-sitosterol and CPP showed the higher TEER increase, with increased values of 37 and 39 Ω·cm² respectively, compared with the cholesterol in different formulations for the potential oral administration test (except the already mentioned outlier formulation D).

3.5. Size measurement of AIF in the donor chamber

The positive control of micelles in the artificial intestine fluids (AIF) were analyzed by atomic force microscopy (AFM) and a dynamic light scattering (DLS). The micelles in the positive control of AIF (see Table 1) was successfully attached to the APTES modified silicon wafer with OsO₄ fat staining and observed by AFM (Fig. 4A).
The largest size population of nanoparticles was less than 10 nm and the mean size of micelles was $14.9 \pm 0.57$ nm (Mean ± SE, N=388) that were over evaluated as described in discussion. Fig. 4B showed the particle size distribution from the dynamic light scattering (DLS), the mean value being $2.3 \pm 0.4$ nm (Mean ± polydispersity of DLS, N=3, Table 1, positive control). There was a difference between AFM observations and DLS measurements but the largest population of the nanoparticles were less than 10 nm in diameter in both measurements.

3.6. Sterol transport analysis and apparent permeability coefficient

Cholesterol transport through the intestine cell model system was evaluated by measuring the cholesterol concentration in the AIF and in the acceptor chambers by HPLC. The amount of transported cholesterol and β-sitosterol was then quantified. The positive control added in the donor chamber (0.5 ml of 0.1 mM of cholesterol) showed the highest cholesterol transport into the acceptor chamber with a value of $0.055 \pm 0.028$ μg (Mean ± SE, N=3, Fig. 5). In the first negative control (buffer, Neg. Cont. I), cholesterol was not detected in the acceptor chamber. The other negative control of bile salt (Neg. Cont. II) showed transport cholesterol in the acceptor chamber as $0.008 \pm 0.008$ μg (Mean ± SE, N=3). In the third negative control of bile salt/monoglyceride/oleic acid in donor chamber (Neg. Cont. III), the cholesterol was detected in the acceptor chambers, as $0.01 \pm 0.01$ μg (Mean ± SE, N=3). No cholesterol was detected in the acceptor chamber in the sample with the 0.1 mM of cholesterol and 0.1 mM of β-sitosterol in the donor chamber. In the presence of CPP with cholesterol in the donor chamber, the cholesterol was detected as $0.011 \pm 0.011$ μg (Mean ± SE, N=3), about the same cholesterol transport value as of two negative controls (Neg. Cont. II and Neg. Cont. III) as shown in Fig. 5.

Based on the initial cholesterol concentration in the donor chamber from positive control, the apparent permeability coefficient ($P_{\text{app}}$) was calculated. The cholesterol $P_{\text{app}}$ of the positive control was $0.33 \pm 0.17 \times 10^{-6}$ cm/s (Mean ± SE, N=3). The $P_{\text{app}}$ of the β-sitosterol + cholesterol co-micelle test was 0 and the $P_{\text{app}}$ of the CPP + cholesterol co-micelle test was calculated as $0.06 \pm 0.06 \times 10^{-6}$ cm/s (Mean ± SE, N=3) (Table.3). The cholesterol transport inhibition was shown by the values of the $P_{\text{app}}$ of cholesterol in Fig. 6. In the potential oral administration test, most of formulations (A, B, C, and F) showed an effect on cholesterol transport with values of
$P_{\text{app}}$ from 0 to $0.09 \times 10^{-6}$ cm/s. However, formulation D and E increased the cholesterol transport more than the positive control.

### 3.7. Cholesterol and β-sitosterol absorption in the intestine cells

β-sitosterol was not detected in any of the analyzed acceptor chambers, even in the acceptor chambers of formulation D and E that showed higher cholesterol transport than positive control. However, β-sitosterol was detected in the intestinal cells, which were collected after three times washing with HBSS++. In order to investigate the absorption correlation between cholesterol and β-sitosterol, the cell permeability coefficient ($P_c$) of cholesterol and β-sitosterol was calculated (Table 3). $P_c$ expresses the sterol absorption from the donor chamber (apical region) into the intestinal cells. In the cholesterol positive control (cholesterol in MG/OA micelles with bile salt), cholesterol $P_c$ was found to be $17.64 \pm 0.70 \times 10^{-6}$ cm/s (Mean ± SE, N=3). In the presence of β-sitosterol, the analyzed cholesterol $P_c$ increased to $24.55 \pm 7.64$ (x $10^{-6}$ cm/s). By comparison of cholesterol $P_c$ and β-sitosterol $P_c$, the presence of β-sitosterol did not seem to have an effect on the cholesterol absorption in the intestinal cells. In the presence of β-sitosterol only, the flux of cholesterol to the cells was increased 1.34 times but in the presence of commercial pine-derived phytosterol (CPP), the cholesterol flux was decreased 2.2 times compared with the positive control (Table. 3). In the potential oral administration formulation test, the cholesterol flux into the intestine cells was lower than 23% in average. More β-sitosterol (1.3-13.5 times) was detected in the intestinal cells in the potential oral administration test of CPP in comparison with the co-micelle test of β-sitosterol ($β$-sitosterol $P_c$ was $0.54 \pm 0.04 \times 10^{-6}$ cm/s, Mean ± SE, N=3) and CPP ($β$-sitosterol $P_c$ was $0.94 \pm 0.19 \times 10^{-6}$ cm/s, Mean ± SE, N=3). However, the amount of β-sitosterol absorbed in the intestine cells was not relevant to the cholesterol permeability to acceptor chamber in any case (Table 3).

### 4. Discussion

The HT29-MTX cell growth profile was different from the typical sigmoidal behavior of animal cell line cultures. The cells were continuously growing up to three fold ($15.5 \times 10^5$ cells/cm²) over its confluence in 15 days of culture (Fig. 2A). After 15
days of the culture, the cell growth curve was fluctuating and the TEER value dropped hugely (150 Ω·cm²) from 425 Ω·cm² in 15 days of culture to 275 Ω·cm² in 16 days of culture. The TEER value was increased again to 400 Ω·cm² in 17 days of culture. This phenomenon indicated a possibility to produce an unstable intestinal model system during continuous cell growing after 15 days of culture. Therefore, the HT29-MTX cells were used after 15 days of culture in this study.

The artificial intestine fluids used in this work were designed and prepared to mimic the absorbable lipid micelles that are produced by in vivo fat metabolism by various lipases (Carey et al., 1983) and the digested fats such as monoglycerides and fatty acids were used accounting for in the developed in vitro system. Therefore, the artificial intestine fluid needs to be composed of the hydrolyzed fats that form absorbable micelles to enter enterocytes (Fig. 1). Previously, an in vivo rat intestine fat absorption study showed that the micelles composed of monoglyceride, oleic acids, and 40 mM of bile salt were successfully attached the cell membrane of intestine microvilli brush border throughout the mucus layer (Ashworth and Lawrence, 1966). Ashworth and Lawrence did not observe the transported nano-sized micelles in the cytoplasm but observed membrane thickening as well as found visible intravesicular lipid droplets in the cytoplasm; the digested fat micelles are absorbed into cells but the mechanism is not clear. Accordingly, in the present study, the components of artificial intestinal fluid (AIF) were mixed and combined to form micelles with particle sizes below 20 nm in diameter (Table 1). The digested fats used in the present work were 0.5 mM of monoglyceride and 1 mM of oleic acid (MG/OA) due to solubility of 0.1 mM of cholesterol with 33 mM of sodium taurocholate that is related to lipid digestion and lipophilic micelle formation. In details, the bile salt was used 6-13 times higher concentration compared with previous studies of intestine cell model system (Field et al., 1997; Palmgren et al., 2005; Petruzzelli et al., 2009). The amount corresponds to the physiological condition in the Duodenum and small intestine of the human body because the 3-4 g of bile salt was secreted to Duodenum 8 times a day (around 24-32g per day) and reabsorbed in colonic enterocytes (Heaton, 1969; Hofmann, 1999), and the average volume of human Duodenum, the place of lipid digestion, was reported to be 275.5 ml from 105 people, aged from 13-93 years old (Suman, 2013). Based on these data, bile salt concentration is over 70 mM in the Duodenum and then is diluted by the
volume of the small intestine. The relatively high concentration of bile salt would keep the micelles of absorbable size, 3 nm-25 nm (Ashworth and Lawrence, 1966). The used amount of bile salt in the present work was chosen in a concentration that forms the size of absorbable micelle formation into enterocytes (see Figure in Ref [2]).

In the present study we have shown that the sizes of the most of micelles are less than 20 nm as determined by AFM and DLS. The seemingly larger sizes measured in AFM compared to DLS is likely due to particle flattening upon adhesion, and individual particle widening due to the size of the cantilever tip (~7 nm). Moreover the resolution of the AFM images is around ~5 nm². Therefore the particle size measurement by AFM has a slight tendency to overestimate the size distribution. The largest distribution however is in the size range expected from comparing with DLS (<10 nm). The average size of nanoparticles was consistently less than 20 nm, which is the absorbable size relevant for enterocytes (Fig. 4).

We have used KOH/methanol saponification to isolate sterols (cholesterols and phytosterols) from collected samples as well as to hydrolyze cholesteryl ester that could be synthesized from cholesterol with acylcoenzyme A:cholesterol acyltransferase (ACAT) in the intestinal cells (Chang et al., 2009; Han et al., 2014). In order to discern the cholesterol transport through intestinal cells regardless of bile salt and fat emulsion effect, we have used three negative controls; only HBSS++, buffer, HBSS ++ plus bile salt and HBSS++, and bile salt plus MG/OA. A small amount of cholesterol transport was detected in the bile salt and MG/OA control that could come from a natural cholesterol metabolism stimulated by bile salt and MG/OA. The positive control showed a five-fold higher cholesterol transport than negative controls (Fig. 5).

Subsequently, the commercial pine-derived phytosterols (CPP) inhibited cholesterol transport in the intestine model system. The used CPP contained 77.6 % β-sitosterol, 11.3% β-sitostanol, 6.6% Campesterol, 1.2% Campesterol, 0.7% Stigmasterol, and over 3% of other sterols. β-sitosterol is well-known cholesterol transport inhibitor and the major composition of the CPP. Therefore, we used β-sitosterol as the analytical control and standard to use amount of CPP in the test. The apparent permeability of cholesterol (cholesterol $P_{app}$) showed the perfect cholesterol transport inhibition by β-sitosterol (Fig. 6). In the presence of CPP, the
cholesterol was detected in only one acceptor chamber out of 3 replicates likewise in the second and third negative controls. Therefore, the abnormal value of standard error (SE) of cholesterol $P_{\text{app}}$ of CPP (0.06 ± 0.06 x $10^{-6}$ cm/s (Mean ± SE, N=3)) was calculated because the transported cholesterol was not detected in two acceptor chambers out of three replicates likewise in the two negative controls (Table 3). The data indicated that the CPP blocked the cholesterol transport effectively with cholesterol or similar value of negative controls without added cholesterol. The cholesterol transport in the two negative controls seems to be natural fat metabolism of intestine cells, stimulated by bile salt or added digested fats (monoglyceride and oleic acid). Since CPP is for a commercial supplement to administrate orally, the six different liquid formulations were tested by the mimicking condition of the acidic stomach and the neutral intestine. Four formulations showed clear effects of cholesterol transport inhibition but two formulations even stimulated cholesterol transport (Table 3). The stimulated formulations would not be recommended for oral administration based on the test.

Another interesting result of the present work is the finding of cholesterol transport inhibition by the commercial pine-derived phytosterols (CPP). The CPP includes around 77.6% of β-sitosterol that was considered as a competitor for intestinal cell absorption in the intestinal lumen (Christiansen et al., 2003; Trautwein et al., 2003), but β-sitosterol cell permeability ($P_c$) was not significantly influencing cholesterol absorption in the cells (Table 3). β-sitosterol was not detected in the any of acceptor chambers indicating that β-sitosterol is absorbed in the cell but not transported to the basolateral region. Therefore, the site of cholesterol transport inhibition of phytosterols could be in the intestine cells as reported in previous studies (Brauner et al., 2012; Rozner and Garti, 2006). Considering our finding of an increase of the trans-epithelial electric resistance (TEER) by β-sitosterol and CPP by 37 and 39 Ω·cm², respectively, the present study supports earlier suggestions that the cholesterol transport inhibition is related to stabilization of the cell membrane by phytosterols (Rozner and Garti, 2006).
5. Conclusion

Cholesterol transport was successfully inhibited by β-sitosterol and the commercial pine-derived phytosterols (CPP) in an HT29-MTX intestinal cell model. The CPP supplement was effective to inhibit cholesterol transport in co-micelle formation of an artificial intestine fluid as well as in the potential oral administration test. There are 6 different classified formulations of CPP corresponding to different ways of administration. Four formulations out of six tested formulations displayed a clear effect of cholesterol transport inhibition, while two formulations did not inhibit cholesterol transport. Moreover, cholesterol transport was increased in two formulations (Fig. 6). Therefore, formulation of the CPP seems to be a critical factor for oral administration with the purpose of cholesterol uptake inhibition.

Acknowledgments

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References


Fig. 1. The schemes of the intestinal cell model system for cholesterol transport. Triglycerides, cholesterol ester, and phospholipids are digested by bile salts and lipases in the intestine lumen, and the digested monoglycerides, fatty acids, and cholesterol-containing micelles enter into intestine cells and are transported to the basolateral region in the form of chylomicrons. Fat digestion and absorbable micelle formation in the intestine lumen (left). The digested fat including cholesterol is transported from the apical region to the basolateral region by forming chylomicrons (upper right). The experimental set-up for the intestinal cell model system (lower right). The set-up of the intestinal cell model system for cholesterol transport is also shown (lower right). BA: bile salt, MG: monoglyceride, FA: fatty acid, TG: triglyceride, PS: phospholipid, C: cholesterol, CE: cholesteryl ester.
Fig. 2. Growth curves and development of cell layer trans-epithelial electric resistance (TEER). (A) Cell growth curve of HT29-MTX cells for 20 days in a 12-well cell culture. (B) The variation of TEER of HT29-MTX cells on a filter membrane in the 12 trans-well plates by days of culture. The upper line (black) is the TEER variation of HT29-MTX cells by days of culture; the lower line (red) is the control of TEER. All values were Mean ± SE (N=3).
Fig. 3. Two-dimensional and three-dimensional images from a Z-stack from two-photon confocal laser microscopy of HT29-MTX cell nuclei after 15 days of culture stained by Hoechst 33342. (A) Multilayered structure from two-dimensional (XY-axis, XZ-axis, and YZ axis) images of the cultured cells. (B) Three-dimensional view of Z-stack image (XYZ-axis); the cells were folded likewise intestine tissue.
<table>
<thead>
<tr>
<th>Sample description</th>
<th>Composition of AIF</th>
<th>Size in Dia. (nm)</th>
<th>z-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control I</td>
<td>0.01 M HBSS++</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33 mM sodium taurocholate 0.01 M HBSS++</td>
<td>2.3 ± 0.1</td>
<td>-24.0 ± 1.7</td>
</tr>
<tr>
<td>Negative control II</td>
<td>33 mM sodium taurocholate 0.01 M HBSS++</td>
<td>2.6 ± 0.3</td>
<td>-13.7 ± 3.9</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.1 mM cholesterol 0.5 mM MG/ 1 mM OA 33 mM sodium taurocholate 0.01 M HBSS++</td>
<td>2.3 ± 0.4</td>
<td>-51.2 ± 7.4</td>
</tr>
<tr>
<td>Cholesterol-micelles</td>
<td>0.1 mM β-sitosterol 0.1 mM cholesterol 0.5 mM MG/ 1 mM OA 33 mM sodium taurocholate 0.01 M HBSS++</td>
<td>3.0 ± 0.4</td>
<td>-46.4 ± 2.2</td>
</tr>
<tr>
<td>Cholesterol+CPP* micelles</td>
<td>CPP (0.1 mM β-sitosterol) 0.1 mM cholesterol 0.5 mM MG/ 1 mM OA 33 mM sodium taurocholate 0.01 M HBSS++</td>
<td>3.1 ± 0.4</td>
<td>-44.1 ± 6.7</td>
</tr>
</tbody>
</table>

CPP*: commercial pine-derived phytosterol supplement, 77.6% of β-sitosterol included.
All values were Mean ± PD (polydispersity of DSL), N=3

**Table 1.** Physico-chemical properties of artificial intestine fluid (AIF) and AIF compositions of each donor chamber for the intestine cell model.
<table>
<thead>
<tr>
<th>Sample description</th>
<th>TEER (Ω·cm²) before incubation</th>
<th>TEER (Ω·cm²) after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control I</td>
<td>252 ± 13</td>
<td>260 ± 20</td>
</tr>
<tr>
<td>Negative control II</td>
<td>281 ± 6</td>
<td>298 ± 17</td>
</tr>
<tr>
<td>Negative control III</td>
<td>291 ± 3</td>
<td>311 ± 23</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(only cholesterol micelles)</td>
<td>283 ± 8</td>
<td>348 ± 35</td>
</tr>
<tr>
<td>Cholesterol + β-sitosterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-micelles</td>
<td>279 ± 13</td>
<td>316 ± 16</td>
</tr>
<tr>
<td>Cholesterol + CPP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-micelles</td>
<td>276 ± 15</td>
<td>315 ± 28</td>
</tr>
<tr>
<td><strong>CPP formulations for the</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>potential oral</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>administration test</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol micelles +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation A</td>
<td>290 ± 3</td>
<td>303 ± 4</td>
</tr>
<tr>
<td>Cholesterol micelles +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation B</td>
<td>275 ± 9</td>
<td>303 ± 37</td>
</tr>
<tr>
<td>Cholesterol micelles +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation C</td>
<td>291 ± 15</td>
<td>312 ± 8</td>
</tr>
<tr>
<td>Cholesterol micelles +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation D</td>
<td>265 ± 8</td>
<td>336 ± 3</td>
</tr>
<tr>
<td>Cholesterol micelles +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation E</td>
<td>301 ± 29</td>
<td>328 ± 15</td>
</tr>
<tr>
<td>Cholesterol micelles +</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation F</td>
<td>293 ± 8</td>
<td>314 ± 32</td>
</tr>
</tbody>
</table>

**Table 2.** Trans-epithelial electrical resistance (TEER) before and after 1 hour incubation with artificial intestine fluid in a trans-well set-up of HT29-MTX cells. All values were Mean ± SE (N=3). CPP: commercial pine-derived phytosterol supplement
Fig. 4. Measurements of micelle size by AFM and DLS. (A) AFM observation of the positive control of 388 micelles in the donor chamber (the image in a box in the graph) and the size distribution of nanoparticles from AFM. Size bar: 500 nm (B) The size distribution from DLS of the micelles.
Fig. 5. Cholesterol transport in the presence of phytosterols in a HT29-MTX intestinal cell model system with co-micelle formation of cholesterol and phytosterols. Compared with negative controls, cholesterol transport is inhibited by β-sitosterol and CPP. Neg. Cont.: negative control, CPP: commercial pine-derived phytosterol supplement. All values were Mean ± SE (N=3).
### Table 3

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Cholesterol cell Flux $\times 10^4$ (μg/cm²s)</th>
<th>Cholesterol, $P_c$ $\times 10^6$ (cm/s)</th>
<th>β-sitosterol cell flux $\times 10^4$ (μg/cm²s)</th>
<th>β-sitosterol, $P_c$ $\times 10^6$ (cm/s)</th>
<th>Cholesterol, $P_{app}$ $\times 10^6$ (cm/s)</th>
<th>β-sitosterol, $P_{app}$ $\times 10^6$ (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>only cholesterol</td>
<td>728.92 ± 28.84</td>
<td>17.64 ± 0.70</td>
<td>-</td>
<td>-</td>
<td>0.33 ± 0.17</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol + β-sito</td>
<td>1014.45 ± 315.66</td>
<td>24.55 ± 7.64</td>
<td>38.94 ± 7.95</td>
<td>0.94 ± 0.19</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Cholesterol + CPP</td>
<td>329.04 ± 48.58</td>
<td>7.96 ± 1.18</td>
<td>22.28 ± 1.79</td>
<td>0.54 ± 0.04</td>
<td>0.06 ± 0.06</td>
<td>0</td>
</tr>
<tr>
<td>Co-micelles in intestinal cell model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol + CPP_A</td>
<td>500.27 ± 25.49</td>
<td>12.11 ± 0.62</td>
<td>215.70 ± 26.50</td>
<td>5.2 ± 0.64</td>
<td>0.05 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Cholesterol + CPP_B</td>
<td>617.39 ± 259.67</td>
<td>14.94 ± 6.28</td>
<td>302.05 ± 16.76</td>
<td>7.28 ± 0.40</td>
<td>0.05 ± 0.05</td>
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<tr>
<td>Cholesterol + CPP_C</td>
<td>516.00 ± 63.97</td>
<td>12.49 ± 1.55</td>
<td>52.20 ± 6.96</td>
<td>1.26 ± 0.17</td>
<td>0.09 ± 0.05</td>
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</tr>
<tr>
<td>Cholesterol + CPP_D</td>
<td>540.25 ± 226.20</td>
<td>13.07 ± 5.47</td>
<td>60.33 ± 11.30</td>
<td>1.45 ± 0.27</td>
<td>10.74 ± 5.23</td>
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<tr>
<td>Cholesterol + CPP_E</td>
<td>724.60 ± 86.11</td>
<td>17.54 ± 2.08</td>
<td>65.76 ± 25.98</td>
<td>1.59 ± 0.63</td>
<td>0.89 ± 0.66</td>
<td></td>
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<tr>
<td>Cholesterol + CPP_F</td>
<td>444.12 ± 28.75</td>
<td>10.75 ± 0.70</td>
<td>754.83 ± 80.00</td>
<td>5.51 ± 0.58</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$P_c$: Cell permeability coefficient, hereby sterol absorption from apical region to intestinal cells

$P_{app}$: Apparent permeability coefficient, hereby sterol absorption from apical region to basolateral region

CPP: commercial pine-derived phytosterol supplement

All values were Mean ± SE (N=3).
**Fig. 6.** The cholesterol apparent permeability of the co-micelle test (CHOL+β-sito. and CHOL+ CPP) and the mimic of oral administration test (six CPP formulations, A to E). Compared to the positive control (only CHOL in the graph), the two co-micelle testers as well as the formulation A, B, C, and F show a clear inhibition of cholesterol transport, whereas formulation D and E show increased cholesterol transport. All values were Mean ± SE (N=3). CPP: commercial pine-derived phytosterol supplement.