Effects of Disodium Ascorbyl Phytostanol Phosphates (FM-VP4) on Cholesterol Accumulation Within Rat Intestinal Cells

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ABSTRACT

The objective of this study was to determine whether FM-VP4, a novel compound derived from plant sterols, can effectively reduce cholesterol accumulation within rat intestinal epithelial crypt (IEC-6) cells. IEC-6 cells were cultured in Dulbecco's minimal essential medium (DMEM) containing 5% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1 units/mL insulin at 37°C under a humidified 5% CO₂ atmosphere and seeded at 6.4×10^4 cells/well in 48-well plates. Experiments were initiated 14 days postconfluence. IEC-6 cells were exposed to [³H]cholesterol micelles (containing oleic and taurocholic acids), co-incubated with FM-VP4 (0, 10, 50, and 100 µM) in Hepes Buffered Sterile Saline (HBSS). Cells were also preincubated with FM-VP4 prior to [³H]cholesterol micelle incubation to determine whether its effects are elicited intracellularly. The cellular localization of cholesterol was determined using digitonin. To determine the effects of cholesterol on the extent of FM-VP4 accumulation within IEC-6 cells, [³H]FM-VP4 was incubated with IEC-6 cells in the presence of unlabeled cholesterol micelles (0, 10, and 50 µM). The extent of [³H]cholesterol or [³H]FM-VP4 associated with cell monolayers was determined after cell lysis

using liquid scintillation counting in a Beckman LS6500 Scintillation Counter. Dose-response and time course studies were performed in which control (no FM-VP4 treatment) and FM-VP4 (10-100 µM) were co-incubated with 50-µM [³H]cholesterol micelles from 1 minute to 24 hours. Incubation with only 50-µM FM-VP4 for less than 24 hours resulted in a 50% to 60% reduction ($n = 6$, $P < .05$) in [³H]cholesterol associated with the monolayer compared with control ($n = 6$). Preincubation of FM-VP4 did not elicit a significant reduction in cholesterol accumulation compared with control ($n = 6$). Approximately 25% of the total [³H]cholesterol associated with the cells was determined to be cytosolic, while 75% was noncytosolic in the presence and/or absence of FM-VP4. [³H]FM-VP4 was also shown to associate with IEC-6 cells at similar concentrations to cholesterol with the most pronounced inhibition of FM-VP4 accumulation occurring at a cholesterol concentration of 50 µM. However, cholesterol-induced inhibition was detectable only after 1 hour of incubation. FM-VP4 inhibits cholesterol accumulation within IEC-6 cells and is most effective at equimolar concentrations with cholesterol. Our findings further suggest that the action of FM-VP4 is likely at the cell surface and not elicited intracellularly.

KEYWORDS: IEC-6 cells, phytosterols, cholesterol accumulation, cytotoxicity

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INTRODUCTION

Cardiovascular disease is a major cause of death in North America. It has been estimated that nearly half of all annual deaths in the United States can be attributed to coronary artery disease.¹ The cost of this disease in the United States is staggering, at about US \$50 billion to \$100 billion per

Figure 1. Structure of FM-VP4.

year in medical expenses and lost wages.¹ Risk factors for cardiovascular disease include gender, age, genetic predisposition, obesity, smoking, hypertension, diabetes, and high serum low-density lipoprotein-cholesterol and triglycerides levels. In clinical terms, a patient is considered hypercholesterolemic if his/her total cholesterol in plasma is greater than 240 mg/dL (6.2 mmol/L).² Normal healthy adults should aim for less than 200 mg/dL cholesterol in serum to be considered healthy.² High levels of cholesterol in serum have been shown to accelerate the formation of atherosclerotic plaques, a condition marked by hardening of the arteries. As these plaques grow, their rough surface can trigger the formation of a blood clot (thrombus) and inhibit blood flow.² If the thrombus forms within blood vessels that supply the heart, then a heart attack or myocardial infarction could result.

Recently, our laboratory has developed a water-soluble phytostanol analogue, disodium ascorbyl phytostanyl phosphates, FM-VP4 (**Figure 1**), that has been shown to decrease plasma cholesterol and LDL-cholesterol levels and cholesterol gastrointestinal absorption in vivo.³⁻⁵ FM-VP4 is derived from sitostanol and campestanol, natural sterols found in abundance within plants. Currently, the mechanism of action of FM-VP4 is not fully understood. We have previously published studies demonstrating that FM-VP4 decreases cholesterol accumulation in Caco-2 cells.⁶ However, the limitation of this study was the use of a cell line that mimics a region of the gastrointestinal tract (ie, colon) where cholesterol is not absorbed. In addition, in those investigations we could not distinguish between cell membrane-associated cholesterol and intracellular cholesterol accumulation. Both of these limitations will be addressed in the current studies.

The studies presented in this manuscript were designed to provide further information on how FM-VP4 may inhibit the uptake of cholesterol into rat intestinal epithelial crypt cells (IEC-6). The main objective of this study was to determine whether FM-VP4 could effectively reduce cholesterol accumulation within rat intestinal IEC-6 cells.

IEC-6 cells are derived from rat small intestinal crypt region and represent an intestinal in vitro model.^{7,8} This model is used to investigate the effects of FM-VP4 in a cell line with duodenal and jejunal functional characteristics, because the majority of cholesterol is absorbed in the duodenum and jejunum of the human gastrointestinal tract.⁹

Our hypothesis is that inhibition of cholesterol uptake by FM-VP4 should be maximal at equimolar concentrations with [³H]cholesterol, due to our hypothesis that FM-VP4 engages in direct competition with cholesterol for uptake into the epithelial cell.

MATERIALS AND METHODS

Chemicals

FM-VP4 primarily comprises 2 molecular entities, campestanol and sitostanol, each covalently linked to ascorbic acid by a phosphodiester bond (campestanol:sitostanol, 34.62:62.41 %wt/wt) (**Figure 1**). Cell culture media and reagents (Delbecco's minimal essential medium [DMEM], insulin, penicillin/streptomycin solution, fetal bovine serum [FBS], phosphate buffered saline [PBS], trypsin 0.25% with EDTA 1mM) were acquired from Gibco Corp (Grand Island, NY). Tissue culture flasks were from Corning Costar (Cambridge, MA), and 48-well tissue culture plates were from Nalge Nunc (Rochester, NY). [³H]cholesterol (1 mCi/mL, 45 Ci/mmol) was from Amersham Pharmacia Biotech (Arlington

Heights, IL). [^3H]FM-VP4 (0.946 mCi/mL, 689 mCi/mmol) was from Chem-Syn Labs (St. Louis, MO). Cholesterol was obtained from Sigma Chemicals (St. Louis, MO). FM-VP4 (Lot # 2670A-1P) was obtained from Forbes Medi-Tech (Vancouver, British Columbia, Canada). KCl, KH_2PO_4 , NaCl, and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ were obtained from BDH Supplies (Dorset, England). Oleic acid, sodium taurocholate, sodium deoxycholate, digitonin, Triton-X 100 were from Sigma Chemicals.

Cell Culture

The rat small intestinal epithelial crypt cell line IEC-6 (Cat. #CRL-1592) was acquired from American Type Culture Collection (Rockville, MD). The cells (passage cell number 14-23) were seeded at 10 000 cells/cm² and subcultured every 7 days using trypsin 0.25% with EDTA 1 mM. The growth medium was changed every 2 to 3 days. For experiments, IEC-6 cells were seeded onto 48-well plates at 80 000 cells/cm² and grown for 12 to 16 days postconfluence before using.

Cells were maintained in DMEM containing 5% heat-inactivated fetal bovine serum (HI-FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1 units/mL bovine insulin at 37°C under a humidified 5% CO₂ atmosphere. Complete medium was aseptically filtered into a new 500-mL glass bottle and stored at 4°C until used.

Effect of FM-VP4 on [^3H]Cholesterol Uptake

Dose-response and time-course

[^3H]cholesterol cell accumulation was determined following preincubation for 1 to 60 minutes with FM-VP4 (50 µM). The effects of FM-VP4 were also compared with a known detergent, sodium deoxycholate (50 µM). At the end of the preincubation period, FM-VP4 was washed away using ice-cold PBS containing 0.1% fatty acid-free BSA. [^3H]cholesterol was then added at a concentration of 50 µM and incubated for 1 hour. HBSS was collected and cells were washed with ice-cold PBS containing 0.1% fatty acid-free BSA. The monolayer was lysed with 1% Triton-X 100 in HBSS and counted for radioactivity using liquid scintillation in a Beckman LS6500 Scintillation Counter. The percentage of [^3H]cholesterol associated with cell monolayers was determined from the ratio of radioactivity in the lysate to that in the external

standard. The external standard contained the total amount of radioactivity incubated with the cells.

Distinguishing cytosolic and noncytosolic accumulation

Cytosolic and noncytosolic accumulation of [^3H]cholesterol was distinguished after treatment with digitonin as previously described.¹⁰ Rat IEC-6 cells were co-incubated with [^3H]cholesterol micelles (50 µM) and increasing concentrations of FM-VP4 (10-50 µM) in HBSS for 30 minutes. Control groups were not exposed to FM-VP4. At the end of 30 minutes, the cell membrane was selectively perforated using a 0.1875-mg/mL ice-cold digitonin solution for 5 minutes with gentle agitation. The solution was carefully removed and counted for radioactivity at the end of 5 minutes. The monolayer was then lysed with 1% Triton-X 100 in HBSS and counted for radioactivity similar to the protocol mentioned above.

Effect of Cholesterol on [^3H]FM-VP4 Uptake

[^3H]FM-VP4 (50 µM) was incubated with IEC-6 cells for 1 minute to 1 hour in the presence of unlabeled cholesterol (10 and 50 µM). Control groups were not exposed to cholesterol-containing micelles. In addition, [^3H]FM-VP4 (50 µM) was co-incubated with micelles that did not contain cholesterol. Enough radiolabeled and 5.0255 mg nonradiolabeled FM-VP4 were mixed together in distilled water to generate a 50-µM solution, with a final radioactivity of 1.5 µCi/mL. At the end of the incubation, HBSS was collected and cells were washed with ice-cold PBS containing 0.1% fatty acid-free BSA. The monolayer was lysed with 1% Triton-X 100 in HBSS and counted for radioactivity using liquid scintillation in a Beckman LS6500 Scintillation Counter. The percentage of [^3H]FM-VP4 associated with cell monolayers was determined from the ratio of radioactivity in the lysate to that in the external standard. The external standard contained the total amount of radioactivity incubated with the cells. For all studies, the accumulation of [^3H]cholesterol or [^3H]FM-VP4 was normalized to cellular protein values.

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test using Instat2 (Graph Pad Inc, San Diego, CA). Differences were considered significant if the *P*-value was less than .05.

RESULTS AND DISCUSSION

Effect of FM-VP4 on [3 H]Cholesterol Uptake

Dose-response and time-course

Dose-response and time-course studies were performed in which control (no FM-VP4 treatment) and FM-VP4 treatment (10-100 μ M) were co-incubated with 50 μ M [3 H]cholesterol from 1 minute to 24 hours. Incubation with 50 μ M FM-VP4 for 15 minutes resulted in a 60% reduction in [3 H]cholesterol associated with the monolayer compared with control (Figure 2A).

Dose-response and time-course studies were performed in which control (no FM-VP4 treatment) and FM-VP4 treatment (10-100 μ M) were co-incubated with 50 μ M [3 H]cholesterol from 1 minute to 24 hours. Incubation with 50 μ M FM-VP4 for 15 minutes resulted in a 60% reduction in [3 H]cholesterol associated with the monolayer compared with control (Figure 2A).

The amount of inhibition of cholesterol accumulation by 50 μ M FM-VP4 remained at 50% to 60% at 1 to 4 hours of incubation (Figure 2B). The greater effect of FM-VP4 at inhibiting cholesterol accumulation at 50 μ M compared with 10 μ M is consistent with data collected at 30 to 60 minutes (Figure 2A). In all treatment groups, the total amount of cholesterol accumulated did not increase significantly after 1 hour of incubation. The effect of FM-VP4 at high doses (up to 100 μ M) and prolonged exposure time (up to 24 hours) was also determined. There was no detectable decrease in [3 H]cholesterol accumulation when IEC-6 cells were exposed to 100 μ M of FM-VP4 with 50 μ M [3 H]cholesterol for 2 hours (data not shown), whereas about 60% inhibition was detectable if 50 μ M of FM-VP4 was used (Figure 2B) compared with controls. There was a significant increased accumulation of [3 H]cholesterol if IEC-6 cells were exposed to 50 μ M of cholesterol and 50 μ M of FM-VP4 for 24 hours (data not shown) compared with controls. It should be noted that sitostanol ascorbate and campestanol ascorbate alone did not significantly decrease [3 H]cholesterol accumulation compared with nontreated controls at 1 to 50 μ M (data not shown).

Preincubation and sodium deoxycholate control

[3 H]cholesterol cellular accumulation was determined following preincubation for 1 to 60 minutes with FM-VP4 (50 μ M). The effects of FM-VP4 were also compared with a known detergent, sodium deoxycholate (50 μ M). Preincubation of FM-VP4 did not result in a significant reduction in cholesterol

Figure 2. (A) Percentage accumulation of [3 H]cholesterol following co-incubations with a range of FM-VP4 concentrations. Samples were incubated for 1, 15, 30, and 60 minutes at 37°C and 5% CO₂. Data presented as mean $\pm$ standard deviation, n = 3 for each treatment group. The asterisk indicates that $P < .05$ for that sample, compared with control (0 μ M). Total recovery of [3 H]cholesterol was greater than 96% for all groups. (B) Percentage accumulation of [3 H]cholesterol following co-incubations with a range of FM-VP4 concentrations. Samples were incubated for 1, 2, 3, and 4 hours at 37°C and 5% CO₂. Data presented as mean $\pm$ standard deviation, n = 3 for each treatment group. The asterisk indicates that $P < .05$ for that sample, compared with control (0 μ M). Total recovery of [3 H]cholesterol was greater than 87% for all groups.

accumulation compared with control (0 μ M FM-VP4) (Figure 3). In addition, treating the cells with 50 μ M sodium deoxycholate did not elicit a significant reduction in cholesterol accumulation, compared with control (0 μ M FM-VP4).

Figure 3. Percentage accumulation of [³H]cholesterol following preincubations with 50 μM FM-VP4 concentrations. Samples were preincubated with FM-VP4 for 1, 15, 30, and 60 minutes then incubated with 50-μM [³H]cholesterol micelles at 37°C in humidified 5% CO₂. The treatment group "No preincubation" acted as the control. Data presented as mean ± standard deviation, n = 6 for each treatment group. Total recovery of [³H]cholesterol was greater than 97% for each group.

Figure 4. Percentage accumulation of [³H]cholesterol following co-incubations with a range of FM-VP4 concentrations. Samples were incubated for 30 minutes at 37°C and 5% CO₂. For "Intracellular" and "Membrane associated," the cells were treated with a digitonin perforation solution, and the intracellular and membrane-associated [³H]cholesterol reported respectively. For "No digitonin treatment," the cells were not treated with digitonin and the monolayer was lysed similar to the other cholesterol uptake experiments. Data presented as mean ± standard deviation, n = 3 for each treatment group. The asterisk indicates that *P* < .05 for that sample, compared with control (0 μM). Total recovery of [³H]cholesterol was greater than 94% for all groups.

Distinguishing cytosolic and noncytosolic accumulation

Cytosolic and noncytosolic accumulation of [³H]cholesterol was distinguished after treatment with digitonin.¹⁰ In the control group (0 μM VP4), the distribution of cytosolic [³H]cholesterol was 25% (1.81 ± 0.07) and noncytosolic [³H]cholesterol was 75% (5.60 ± 1.39) (**Figure 4**). This proportionate distribution appears to be consistent in other treatment groups as well, with 10 μM and 50 μM VP4. Overall, the inhibition of [³H]cholesterol accumulation in the treatment group that was not treated with digitonin was 22.1% and 35.9% at 10 μM VP4 and 50 μM VP4, respectively. For all the [³H]cholesterol accumulation studies, greater than 90% of the original [³H]cholesterol incubated was recovered.

Effect of Cholesterol on [³H]FM-VP4 Uptake

[³H]FM-VP4 was shown to associate with IEC-6 cells at similar concentrations to cholesterol with the most pronounced inhibition of FM-VP4 accumulation occurring at a cholesterol concentration of 50 μM, compared with control. However, this cholesterol-induced inhibition was detectable only after 1 hour of inhibition (**Figure 5**). Furthermore, micelles not containing cholesterol did not significantly alter [³H]FM-VP4 cell accumulation compared with controls (data not shown). For all the [³H]FM-VP4 accumulation studies, greater than 90% of the original [³H]FM-VP4 incubated was recovered.

Figure 5. Percent accumulation of [³H]FM-VP4 following co-incubations with a range of nonradioactive cholesterol concentrations. Samples were incubated for 1, 15, 30, and 60 minutes at 37°C and 5% CO₂. Data presented as mean ± standard deviation, n = 6 for each treatment group. The asterisk indicates that *P* < .05 for that sample, compared with control (0 μM). Total recovery of [³H]FM-VP4 was greater than 85% for each group.

DISCUSSION

The purpose of this study was to determine the inhibitory effects of FM-VP4 on cholesterol accumulation within IEC-6 cells. Determining the effects of FM-VP4 in IEC-6 cells would be valuable because IEC-6 cells are derived from the duodenal and jejunal region of the rat small intestine.⁷ In the human gastrointestinal tract, dietary cholesterol is absorbed at these sites.² Therefore, IEC-6 cells may be a physiologically relevant model for cholesterol uptake studies. It should be noted, however, that IEC-6 cells are of crypt cell origin.⁷ They do not fully differentiate without biological stimulation such as basement membrane extract and may not possess the same functional capacity of mature enterocytes.¹¹

The inhibition of cholesterol uptake by FM-VP4 is most effective (60% inhibition) at equimolar concentrations with cholesterol after 15- and 30-minute incubation (**Figure 2A**). The amount of cholesterol accumulation increases for all treatment groups at incubations of less than 60 minutes, the difference being that the treatment groups exposed to FM-VP4 have less accumulation compared with groups not exposed to FM-VP4. This indicates that FM-VP4 does not completely inhibit cholesterol accumulation but rather slows the rate at which it is accumulated. In addition, the extent of cholesterol inhibition does not significantly change with incubation times longer than 1 hour (**Figure 2B**). Significant inhibition of cholesterol accumulation was not observed when the cells were exposed to high doses of FM-VP4 at 100 μ M, or at prolonged incubation time of 24 hours (data not shown). This is likely the result of FM-VP4-induced cellular toxicity, as FM-VP4 has the potential to act as a detergent. However, it could be a normal physiological response by the cell to exposure to FM-VP4, detectable only either at high doses or prolonged incubation times. Further studies are required to determine why cytotoxicity occurs. The lack of inhibition of cholesterol uptake after preincubation of FM-VP4 indicates that FM-VP4 most likely acts at the cell surface to physically prevent cholesterol from being taken up into the cell. If FM-VP4 acted intracellularly, then preincubation with FM-VP4 would result in increased FM-VP4 accumulation inside the cell and, subsequently, a more pronounced inhibitory effect. As shown in **Figure 3**, this effect is clearly not the case.

To determine if FM-VP4 elicited its effects nonspecifically as a detergent, the accumulation of [³H]cholesterol was determined in the presence of

either FM-VP4 or the detergent sodium deoxycholate. Deoxycholate was chosen as the representative detergent as it has a similar structure to the stanol backbone of FM-VP4. Sodium deoxycholate did not inhibit accumulation of cholesterol compared with 50 μ M FM-VP4 (**Figure 3**), suggesting that the mode of action of FM-VP4 is not due to a nonspecific detergent effect.

To determine the effects of FM-VP4 on the cellular localization of cholesterol, cytosolic and noncytosolic [³H]cholesterol was quantified after exposing cells to FM-VP4 using digitonin permeabilization. Digitonin interacts specifically with β -hydroxysterols and selectively perforates the plasma and outer mitochondrial membrane, leaving the inner mitochondrial membrane intact.¹⁰ After treatment with digitonin, the cytosolic contents leak out of the permeabilized membranes while the overall cell structure is maintained.¹² It was assumed that the radioactivity in solution after digitonin treatment was entirely cytosolic [³H]cholesterol, and the remaining radioactivity (after Triton-X 100 treatment) was noncytosolic (ie, plasma membrane-, nuclear-, and ribosomal-associated) [³H]cholesterol. Approximately 25% of the total [³H]cholesterol associated with the cells was determined to be cytosolic (**Figure 4**), while the remaining 75% was noncytosolic, regardless of FM-VP4 treatment. Exposure of cells to increasing concentration of FM-VP4 resulted in decreasing total [³H]cholesterol accumulation. These results do not confirm the site of action of FM-VP4, as there is no statistically significant difference in the cellular localization of [³H]cholesterol in the absence or presence of FM-VP4.

If the site of action of FM-VP4 is at the cell surface, then it may compete with cholesterol for binding sites and therefore inhibit cholesterol absorption. To test this hypothesis, the effects of unlabeled cholesterol on [³H]FM-VP4 accumulation were determined. As shown in **Figure 5**, maximal inhibition of [³H]FM-VP4 accumulation was observed at an equimolar concentration of cholesterol. While this effect is also seen with decreased cholesterol uptake in the presence of FM-VP4, the length of time required to detect a significant decrease in accumulation of radiolabeled FM-VP4 is longer for unlabeled cholesterol (60 minutes for 62% reduction in [³H]FM-VP4 accumulation [**Figure 5**] versus 15-30 minutes for 60% reduction in [³H]cholesterol accumulation [**Figure 2A**]). This could be caused by a reduced affinity of surface receptors for FM-VP4 compared with cholesterol. However, future studies are warranted to test this hypothesis.

The mode of action of FM-VP4 is still unclear. It acts to rapidly reduce accumulation of cholesterol in cultured rat small intestinal crypt cells, likely at the cell surface. The

data suggest that FM-VP4 is a competitive inhibitor of cholesterol uptake, as cholesterol has been shown to reduce accumulation of radiolabeled FM-VP4. FM-VP4 may inhibit the interaction of cholesterol or cholesterol micelles with receptors on the cell surface, or it may act as an antagonist of cholesterol influx channels such as MDR1.^{13,14} Further studies are required to confirm this hypothesis.

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