Measuring the Hypolipidemic, Atheroprotective and Mechanistic Properties of FM-VP4 in LDL-Dominant Animal Models

by

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B.Sc.(Hon.), Trinity Western University, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE in THE FACULTY OF GRADUATE STUDIES (Department of Pathology and Laboratory Medicine)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August, 2003

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Vancouver, Canada

Date: October 9, 2003
ABSTRACT

It is well established that high plasma cholesterol levels play a key role in the development of atherosclerosis, a form of coronary heart disease (CHD). Data from numerous laboratory experiments, genetic and epidemiological studies as well as extensive clinical trials have shown that reducing plasma cholesterol concentrations leads to a decreased risk for the development of CHD. Among the recommended strategies for achieving and maintaining lower plasma cholesterol levels is the use of pharmacological agents. While there are many different classes of lipid-lowering drugs currently available with varying degrees of safety and efficacy, the HMG-CoA reductase inhibitors (statins) are the most widely prescribed for lowering plasma total and LDL-cholesterol levels. HMG-CoA reductase inhibitors, however, are not effective in all individuals and there are safety concerns over the long-term administration of the high doses of these drugs that are often required to achieve target cholesterol levels. FM-VP4 is a novel phytostanol analogue which has been shown to lower plasma cholesterol levels in a variety of animals models. Additionally, FM-VP4 has also demonstrated the ability to reduce the formation of atherosclerotic lesions. The purpose of this study was to measure the cholesterol-lowering and lesion-reduction properties of FM-VP4 as well as to try to ascertain its molecular mechanism of action in LDL-dominant animal models. From our studies in the rabbit model we were unable to make any significant conclusions due to the great variability in response to both dietary cholesterol and FM-VP4 treatment in the different groups of animals over the 10-week treatment period. In the apo B-100 mouse model, however, we demonstrated a 61% decrease in plasma total cholesterol levels as well as a 34% decrease in plasma apolipoprotein B levels compared to control animals on the same high fat, high cholesterol diet. We also showed a small, but significant, decrease in fatty lesion area in the aortic arch of the treated mice compared to controls. In examining the expression of genes involved in cellular cholesterol control (ABCA1, ABCG5, ABCG8 and LDL receptor) we demonstrated a significant decrease in the expression of ABCG5 in the liver of treated animals. Additionally, we showed a trend towards lowering of ABCA1 and ABCG8 in the liver and small intestine of FM-VP4-treated animals. The expression of the LDL receptor in the liver of the mice was unchanged subsequent to FM-VP4 treatment while there was a trend towards increased expression of this gene in the small intestine. These results have led us to propose that the effect of FM-VP4 on the expression of these genes is secondary to its lipid-lowering capabilities.
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<tr>
<td>4S</td>
<td>Scandinavian Simvastatin Survival Study</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette transporter</td>
</tr>
<tr>
<td>AFCAPS/TexCAPS</td>
<td>Air Force/Texas Coronary Atherosclerosis Prevention Study</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine amino transferase</td>
</tr>
<tr>
<td>apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate amino transferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adult Treatment Panel</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BARI</td>
<td>Bypass Angioplasty Revascularization Investigation</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CARE</td>
<td>Cholesterol and Recurrent Events</td>
</tr>
<tr>
<td>CCCC</td>
<td>Canadian Consensus Conference on Cholesterol</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>CL/F</td>
<td>clearance of drug from the systemic circulation as a function of bioavailability (F)</td>
</tr>
<tr>
<td>FDB</td>
<td>familial defective apolipoprotein B-100</td>
</tr>
<tr>
<td>FH</td>
<td>familial hypercholesterolemia</td>
</tr>
<tr>
<td>FM-VP4</td>
<td>disodium ascorbyl phytostanol phosphates</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HDL-C</td>
<td>high density lipoprotein cholesterol</td>
</tr>
<tr>
<td>HL</td>
<td>hepatic lipase</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>liquid chromatography/mass spectrometry/mass spectrometry</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecithin-cholesterol acyl transferase</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LDLr</td>
<td>low density lipoprotein receptor</td>
</tr>
<tr>
<td>LIPID</td>
<td>Long-Term Intervention with Pravastatin in Ischaemic Disease</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>LRC-CPPT</td>
<td>Lipid Research Clinics Coronary Primary Prevention Trial</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NCEP</td>
<td>National Cholesterol Education Program</td>
</tr>
<tr>
<td>NHLBI</td>
<td>National Heart, Lung and Blood Institute</td>
</tr>
<tr>
<td>PBGD</td>
<td>porphobilinogen deaminase</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SR-B1</td>
<td>scavenger receptor-B1</td>
</tr>
<tr>
<td>TC</td>
<td>total cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TLC</td>
<td>therapeutic lifestyle changes</td>
</tr>
<tr>
<td>Vd/F</td>
<td>volume of distribution as a function of bioavailability (F)</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>WGHOD</td>
<td>Working Group on Hypercholesterolemia and Other Dislipidemias</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe Heritable Hyperlipidemia</td>
</tr>
<tr>
<td>WOSCOPS</td>
<td>West of Scotland Coronary Prevention Study</td>
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</tbody>
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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Haydn Pritchard, for taking me on as a graduate student in spite of my kayaking skills, for supporting me in my goals and for teaching me to be a discerning scientist. I would also like to thank my supervisory committee members for their help and direction – even in the small details of my work. Thanks to my colleagues at Forbes Medi-Tech and to the ASL crew for helping to keep my days interesting. Finally to my family: thanks for the love, support, encouragement and money.
1. INTRODUCTION

"The 'cholesterol hypothesis' is no longer a hypothesis. There is no doubt that abnormal cholesterol levels cause major morbidity and mortality and that aggressive treatment saves lives" (Lauer & Fontanarosa, 2001).

1.1. LIPIDS AND LIPOPROTEINS: THE BACKGROUND

1.1.1. Cholesterol

Cholesterol is a structural membrane lipid which is composed of a cyclic 4-ring nucleus with a polar hydroxyl group and a hydrocarbon side chain. Insertion into the eukaryotic plasma membrane is made possible by its amphipathic nature with this polar head and non-polar body. Cholesterol also serves as a precursor molecule to bile acids, corticoids, sex hormones and vitamin D-derived hormones. This sterol is transported through the bloodstream by discreet lipoprotein particles composed of, as their name implies, both lipids and proteins. Five major classes of lipoproteins are found in serum, into which lipoproteins are classified based on their hydrated density. These classes however, are not mutually exclusive as the lipoprotein populations are in a constant dynamic state whereby both their lipid and protein components are being transported into or out of the lipoprotein or exchanged with the components of other lipoproteins.

Cholesterol is transported in the blood by 4 major classes of lipoproteins: very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL) and chylomicrons. Total cholesterol, then, is a measurement of the total cholesterol carried by these four classes of lipoproteins.

1.1.2. Plasma Lipoproteins

1.1.2.1. VLDL

While the largest of the above-mentioned lipoproteins, VLDL carries the least cholesterol of the three. Bearing only 10-15 percent of serum cholesterol, VLDL is a large particle (30-80nm) with a triglyceride-rich core. VLDL also carries several apolipoproteins including
apoB-100, apo C-I, C-II and C-III as well as apoE. VLDL is synthesized in and secreted from the liver and is ultimately the precursor of LDL after a large portion of its triglyceride core is broken down into glycerol and free fatty acids by lipoprotein lipase on endothelial cell surfaces. The initial action of lipoprotein lipase (LPL) and its cofactor apo C-II on VLDL is the production of smaller VLDL remnants and subsequently intermediate density lipoproteins (IDL). A small portion of IDL is then taken up by the liver through the interaction of apo E with the LDL receptor and the remainder is further acted upon by LPL and hepatic lipase (HL) to hydrolyse phospholipids and triglycerides on IDL to produce LDL. Because of their richness in cholesteryl esters, VLDL remnants may play a role in the promotion of atherosclerosis.

1.1.2.2. LDL

LDL cholesterol (LDL-C) comprises 60-70 percent of cholesterol in the blood. LDL particles are classified as having a density of 0.95-1.006 kg/L and bear only a single apolipoprotein: apo B-100. This apolipoprotein serves as the ligand for the LDL receptor-mediated removal of LDL by the liver. LDL's notorious atherogenisity lies in its propensity to become oxidized and subsequently taken up by macrophages.

1.1.2.3. HDL

HDL and its subspecies are the smallest of the lipoprotein classes. Unlike its atherogenic counterparts, HDL is believed to play a cardioprotective role primarily through the action of reverse cholesterol transport. This is a process whereby discoidal, lipid-poor apoA-I-bearing HDL particles are sequestered to peripheral cells (including macrophages) where they function to accept free cholesterol transported across the plasma membrane by the ABCA1 transporter. Spherical HDL particles are then formed as the free cholesterol is esterified with a free fatty acid from lecithin by the action of lecithin-cholesterol acyl transferase (LCAT). About half of the cholesteryl esters from the core of the mature HDL are delivered to the liver and taken up by the HDL receptor: scavenger receptor-B1 (SR-B1). The other 50% are transferred to apoB-containing lipoproteins in exchange for triglycerides by the action of cholesteryl ester transfer protein (CETP).
1.1.2.4. Chylomicrons

Also worth mentioning is the largest of the lipoproteins: the intestinally-derived chylomicron. Chylomicrons are a post-prandial lipoprotein species bearing apolipoprotein B-48 and functioning to transport dietary cholesterol and triglycerides through the bloodstream where they are acted upon by lipoprotein lipase. Apo B-48 is the apolipoprotein product of the intestinal editing of apo B-100 resulting in the translation of only 48% of the apo B transcript.\(^\text{10}\) The significance of this editing may lie in the differences in lipid acquisition between the two apo B species (apo B-100 and apo B-48) as well as the targeting of lipids to their target tissues since, unlike apo B-100, apo B-48 cannot bind the LDL receptor.\(^\text{10}\) Triglyceride-poor, cholesterol-rich chylomicron remnants are subsequently transported into the liver by the LDL receptor-related protein (LRP).\(^\text{5}\) Delayed hepatic clearance of chylomicron remnants may play a role in atherogenesis.\(^\text{11}\)

<table>
<thead>
<tr>
<th>% composition (wt/wt)</th>
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<tbody>
<tr>
<td>Density (g/ml)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Chylomicrons</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>VLDL</td>
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<td>IDL</td>
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<td>HDL</td>
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</table>

Table 1: Major human plasma lipoprotein classes and their properties.

\(TG=\) Triglyceride; \(CH=\) cholesterol; \(PL=\) phospholipid\(^\text{4,6,12}\)
1.2. LDL CHOLESTEROL & CORONARY HEART DISEASE (CHD) – THE ISSUE

Since the first link between cholesterol and atherosclerosis was observed almost one hundred years ago by Russian scientists, Anitschkow and Chalatow when they observed aortic fatty lesions similar to human atheromas after feeding an egg-rich diet to rabbits, the relationship between cholesterol and atherosclerosis has been extensively studied. This relationship has been clearly demonstrated in a variety of animal models as well as in genetic, epidemiological and clinical studies.

1.1.1. Animal Models

1.1.1.1. Rabbit Models

Since Anitschkow and Chalatow, a vast array of atherosclerosis research has been conducted using the cholesterol-fed rabbit as a model of the human disease-state. The draw of this model lies in several factors including the similarity to humans in apolipoprotein B-bearing lipoprotein composition, in lack of hepatic apo B-100 editing and in the abundance of plasma cholesteryl ester transfer protein (CETP) activity. Most importantly, however, is the rabbit's propensity towards developing dietary-induced hypercholesterolemia and subsequent complex atherosclerotic lesions.

Apart from the wild-type New Zealand White Rabbit, several transgenic rabbit strains have been developed to further study different aspects of atherosclerosis. One of these is the Watanabe Heritable Hyperlipidemic (WHHL) rabbit. This transgenic animal possesses a mutation in the gene encoding for the LDL receptor. Such a mutation leads to uninduced hypercholesterolemia, increased serum LDL levels and prominent atherosclerotic lesions. Another transgenic rabbit model is the human apo B-100 rabbit which displays a three-fold increase in plasma cholesterol, most of which is found in the LDL fraction. Conversely, other transgenic rabbit models such as those overexpressing human apo A-I or human lecithin-cholesterol acyl transferase (LCAT) demonstrate increased plasma HDL levels with decreased progression of atherosclerosis.
1.1.1.2. Murine Models

Wild-type mice do not possess CETP and therefore carry about 70% of their plasma cholesterol in HDL. They therefore tend to be resistant to atherosclerosis. For this reason, several strains of transgenic mice have been developed to mimic different aspects of human lipoprotein metabolism and atherogenesis.

The first successful gene knockout model was reported by Zhang et al. in 1992. Chylomicron and VLDL remnants are hepatically cleared through the interaction of apolipoprotein E with the LDL- and chylomicron remnant-receptors. Absence of this protein, therefore, leads to an accumulation of cholesterol-rich remnants in the plasma which are potentially atherogenic. Indeed, in the apoE knockout mouse, there is five times the normal plasma cholesterol levels with accompanying prominent lesions in the proximal aorta by age 3 months, even when animals are fed a low-fat chow. This disease process may be even further accelerated by feeding a high-fat/high-cholesterol diet.

Such a high-fat/high-cholesterol diet is necessary to induce atherosclerosis in several other transgenic mouse models such as the LDL receptor-knockout mouse, the human apo B-100 transgenic mouse and the apo E-Leiden transgenic mouse. All of these models demonstrate varying degrees of hypercholesterolemia and atherosclerosis in response to diets rich in cholesterol and triglycerides.

1.1.1.3. Avian Models

Pigeons are naturally hypercholesterolemic, carrying most of their cholesterol in the HDL fraction. When fed a diet rich in cholesterol and fat, however, White Carneau pigeons develop a different lipoprotein profile, exhibiting significantly increased levels of VLDL and LDL. While this species has been shown to spontaneously develop atherosclerosis, a cholesterol-rich diet and subsequent alteration of plasma lipoprotein profile accelerates the atherosclerotic process in this model.
1.1.1.4. Canine Models

Although dogs are naturally resistant to atherosclerosis, even on low levels of dietary cholesterol, a high-fat/high-cholesterol diet deficient in essential fatty acids has been shown to induce high serum cholesterol levels and a propensity to develop atherosclerosis after one year.\textsuperscript{21}

1.1.1.5. Swine Models

Pigs – both domestic and miniature – have also been extensively studied as models for atherosclerosis because of their similarity to humans in atherosclerotic lesion composition and distribution when fed an atherogenic diet.\textsuperscript{22} On as low as 2\% dietary cholesterol, swine have demonstrated a significant positive association between serum cholesterol concentration and extent of atherosclerotic lesion development.\textsuperscript{23} A particular strain of pigs with inherited hyper-LDL and hypercholesterolemia display spontaneous hypercholesterolemia and, by 3 years of age, complex atherosclerotic lesions.\textsuperscript{24} This further illustrates the link between plasma cholesterol (particularly in the LDL fraction) and atherogenesis.

1.1.2. Genetics

1.1.2.1. Familial Dysbetalipoproteinemia

Also termed Type III Hyperlipoproteinemia, this genetic lipoprotein disorder is primarily characterised by an accumulation of cholesterol-rich chylomicron and VLDL remnants in the plasma.\textsuperscript{25-27} Patients with familial dysbetalipoproteinemia also develop premature and accelerated atherosclerosis. The molecular basis of this disorder lies either in the deficiency of apo E or one of several polymorphic phenotypes (i.e. apo E2/2) which leads to a protein defective in its ability to bind remnant receptors: LDL receptor (LDLr) or LDL receptor-related protein (LRP).\textsuperscript{28} The ensuing hypercholesterolemia and hypertriglyceridemia which distinguish this disorder are a result of several contributing factors. First of all, defects in the ability of apo E to serve as the necessary ligand for binding to remnant receptors leads
to impaired clearance of the intestinally-derived chylomicron remnants and hepatically-derived VLDL remnants often seen as a broad beta band on lipoprotein electrophoresis.\textsuperscript{25} Secondly, apo E functions in the regulation of lipolysis. When functional apo E is absent, triglyceride hydrolysis may be impaired thereby resulting in hypertriglyceridemia. Another contributing factor is the role of apolipoprotein E in VLDL production. Increased apo E has been shown to upregulate hepatic VLDL synthesis, thereby further exacerbating the hyperlipidemia.\textsuperscript{27} In order to clear these lipid-rich remnants from the plasma, an alternate clearing pathway must be employed. This may entail macrophages, ultimately leading to atherosclerosis.\textsuperscript{26}

1.1.2.2. Familial Defective Apolipoprotein B-100

Like familial dysbetalipoproteinemia, familial defective apolipoprotein B-100 (FDB) is a result in the impairment of the binding an apolipoprotein with its receptor. In this case, the ligand is apo B-100 which is the major protein present on LDL. The interaction of LDL with the LDL receptor is regulated by apo B-100 which ultimately controls plasma LDL levels. Since LDL transports 60-70\% of plasma cholesterol, any dysfunction in the ability to clear this lipoprotein from the plasma may lead to an accumulation of cholesterol in the blood and therefore hypercholesterolemia. The clinical presentation of this disorder can display a range of LDL-cholesterol (LDL-C) levels anywhere from 2.7 to 10.3 mmol/L, depending on the functional capabilities of the LDL.\textsuperscript{25, 29} Because the mutation is genetically transmitted co-dominantly, those affected will display a heterogeneous LDL population possessing both LDL that have, as well as those that lack, LDL receptor-binding abilities.\textsuperscript{30} Because of the similarity in mechanism to heterozygous familial hypercholesterolemia, clinical manifestations tend to be very similar and will therefore be discussed in the next section.

1.1.2.3. Familial Hypercholesterolemia

Familial Hypercholesterolemia (FH) is caused by a defect in the LDL receptor gene. In contrast to FDB, it is the receptor that is dysfunctional and not its ligand. In their Nobel Prize-winning research, Brown & Goldstein demonstrated the receptor-mediated endocytotic process through which LDL is bound to the LDL receptor and is subsequently shuttled to the
intracellular lysozome for degradation.\textsuperscript{31} When this process is defective, such as in FH, LDL accumulates in the plasma thereby placing individuals at a very high risk for premature atherosclerosis.\textsuperscript{32} The increase in plasma LDL levels, however, is not solely a result of its impaired clearance via the receptor-mediated pathway. As previously described, LDL is not secreted directly by the liver. Instead, hepatically-derived VLDL delivers triglycerides to extrahepatic tissues through the action of lipoprotein lipase. The resulting triglyceride-depleted particle is IDL. Under normal circumstances, a portion of the IDL is rapidly taken up by the liver while the remainder is subjected to further triglyceride hydrolysis yielding LDL. In the case of the defective LDL receptor, however, IDL is no longer hepatically cleared, thereby leading to an ever larger population of cholesterol-rich LDL particles.\textsuperscript{31}

FH exists in two forms. In the heterozygous form, individuals display an approximately twofold increase in plasma LDL and tend to suffer from myocardial infarctions (MI) by the age of thirty or forty.\textsuperscript{31} In the more severe homozygous form, individuals suffer from six to tenfold increases in plasma LDL levels from birth and may suffer from heart attacks in early childhood. This manifestation of severe atherosclerosis in the absence of any other risk factors provides clear evidence that hypercholesterolemia alone, particularly in the LDL fraction, can directly lead to CHD.

1.1.3. Epidemiology

Because of the well-established link between cholesterol and CHD and the prevalence of CHD throughout many parts of the world,\textsuperscript{33} it is not surprising that several studies have been conducted to further examine this relationship under various environmental and genetic conditions. With the incidence of CHD being so great in middle-aged to elderly North Americans, it is natural to assume that a great deal has been invested into studying the disease in this population. In the Atherosclerosis Risk in Communities (ARIC) study, 12,339 middle-aged participants (45-64 years old) from North Carolina, Mississippi, Minnesota and Maryland with no evidence of CHD were recruited and baseline plasma lipid measurements were taken.\textsuperscript{34} After a ten-year follow-up period, 725 CHD events transpired in this population. The lowest incidence of CHD occurred in individuals with the lowest serum LDL-C measurements. After conducting extensive statistical analysis on the data
obtained from this cohort, researchers concluded that both high serum LDL-C and low serum HDL-C were independent risk factors for CHD. They also demonstrated that for every 1mmol/L increase in serum LDL-C, there was an approximate 40% increased risk of developing CHD.

As well as in initially CHD-free populations, studies have also been conducted on the relationship between serum cholesterol levels and secondary coronary events in patients who have pre-existing coronary artery disease (CAD). In the Bypass Angioplasty Revascularization Investigation (BARI), 1514 patients who had experienced multivessel coronary artery disease were followed for 5 years. From the data collected, they concluded that non-HDL cholesterol levels were a strong and independent risk factor for non-fatal myocardial infarction and angina pectoris in patients with prevalent CAD. It was also demonstrated that for every 0.25mmol/L increase in non-HDL-C, there was a concordant 5% increased risk of myocardial infarction and 10% increase risk of angina pectoris.

While primarily a disease in developed countries, CHD and its associated risk factors is also being increasingly seen in developing countries. In Mexico, for example, the highest serum total cholesterol levels are seen in the northern Mexican states where individuals have a higher income and greater accessibility to animal products. Not surprisingly, the mortality rates from CHD are also highest in these northern states. The authors of this study postulate that the regional differences in hypercholesterolemia and CHD mortality are primarily related to diet. While those in the northern regions have greater access to animal-derived food products, those in the southern states experience increased poverty and malnutrition.

The direct relationship between dietary cholesterol intake and CHD, independent of and in addition to plasma cholesterol levels, has also sparked interest for epidemiologists. In surveying a number of cross-population and within-population studies, Stamler and Shekelle concluded that dietary cholesterol presents an atherogenic potential, independent of its effect on plasma cholesterol concentrations.
While it is rare for children to develop symptoms of atherosclerosis without a genetic predisposition, identifying and modifying risk factors, especially in children with a family history of CAD, may be critical to preventing the disease later in life.\textsuperscript{38} In a recent study in Iran, researchers showed that children of patients suffering from premature myocardial infarction had significantly higher serum levels of total cholesterol, LDL-C and triglycerides as well as significantly lower levels of HDL-C compared to age- and sex-matched controls who did not possess a family history of premature CAD.

From the epidemiological evidence presented here, it is clear that plasma total and LDL cholesterol levels as well as dietary cholesterol intake are positively associated with the development of CHD. This has been demonstrated in healthy populations with no history of CAD, people who have already been treated for CAD, in developing countries where CHD is becoming more and more prevalent, as well as in children who have a first-degree relative with premature CHD.

\textbf{1.2. Reduction of Serum Cholesterol – The Solution}

The evidence presented in animal, genetic and epidemiological studies has led to the formation of different initiatives to provide recommendations and guidelines for the prevention and treatment of CHD in various nations.

\textbf{1.2.1. NCEP ATP III}

Initiated by the National Heart, Lung and Blood Institute (NHLBI) in the United States in 1985, the National Cholesterol Education Program (NCEP) has as its mandate to reduce the number of Americans with high blood cholesterol thereby reducing morbidity and mortality from coronary heart disease.\textsuperscript{39} This is accomplished by the NCEP through educating both the public as well as health professionals regarding the risks of plasma cholesterol levels and CHD and prevention of coronary events through lowering blood cholesterol. As a body, the NCEP has assembled a group of professionals to form the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults.\textsuperscript{9} The function of this panel is to review and analyze clinical trials dealing with lipid lowering and CHD and, based on
their interpretation of the empirical data and clinical intervention evidence, devise appropriate clinical recommendations and guidelines. As new evidence arises from scientific research, the Adult Treatment Panel (ATP) produces clinical updates to reflect the results of such evidence. As such, the most recent report is the third of such reports, ATP III, and was published in 2002.9

1.2.1.1. Guidelines for Primary Prevention

One third of those who suffer from myocardial infarction will die within 24 hours. Those who survive such an event may experience severe consequences such as congestive heart failure, angina, arrhythmias and an increased risk of sudden death.40 For these reasons, primary prevention is necessary to reduce risk factors for coronary heart disease and prevent its development.

ATP III suggests that there are two approaches to primary prevention: population strategies and clinical strategies.9 Population strategies include modification of diet and other lifestyle practices that may pose as a risk for the development of atherosclerosis. Clinical strategies are those strategies above and beyond population strategies that are intended for those at a higher risk of developing CHD and who need more urgent intervention. Clinical primary prevention may then further be separated into long-term and short-term prevention. Long-term prevention is aimed at those who are not currently at high risk of developing atherosclerosis but have a high probability, at some point in their lives, of being at a higher risk of doing so. Long-term prevention includes lifestyle changes of risk factors that are to be implemented and maintained over the course of one’s life in order to prevent atherogenesis. Short-term prevention is aimed at those who, in all likelihood, already have evidence of atherosclerosis and who are at high risk of suffering from an acute coronary event. In addition to lifestyle changes, drug intervention is often required in short-term prevention.

At the outset, it is suggested that an optimal plasma LDL-cholesterol concentration is less than 2.56mmol/L (100mg/dL). However, for those who have greater LDL-C levels, the recommendations put forth by ATP III are very specific to plasma LDL levels as an
independent risk factor. Low levels are considered to be <3.33mmol/L (130mg/dL), borderline high levels are 3.33-4.03mmol/L (130-159mg/dL) and high LDL-C levels are >4.10mmol/L (160mg/dL). In addition to these lipid levels, guidelines as to the necessity of drug intervention are also based on the person's number of independent risk factors as well as the degree of risk which this person is at for the development of atherosclerosis. These guidelines stress that risk reduction using drug intervention needs to be balanced with its cost-effectiveness. In other words, the lower the risk, the more emphasis should be placed on therapeutic lifestyle changes. Another important recommendation made by ATP III in terms of primary prevention is that routine cholesterol testing should begin at the age of twenty in order to help minimise long-term risk of CHD.

1.2.1.2. Guidelines for Secondary Prevention

Secondary prevention addresses prevention of recurrent coronary events in those with established CHD since they are at a very high risk for such recurrence. The ATP III goal for secondary prevention is LDL-C levels <2.56mmol/L (100mg/dL). For this reason, the ATP III recommendation is that people with established CHD should receive pharmacological intervention to achieve this goal. Where persons with established CHD have baseline LDL-C levels ≥3.33mmol/L (130mg/dL), therapeutic lifestyle changes and control of nonlipid risk factors should be introduced alongside drug therapy to achieve the LDL-C goal of <2.56mmol/L. Finally, in patients with established CHD and LDL-C levels of 2.56-3.31mmol/L several alternatives should considered including drug therapy, both for cholesterol lowering and for modification of atherogenic dyslipidemia as well as lifestyle changes and control of nonlipid risk factors.

1.2.2. CCCC

The Canadian Consensus Conference on Cholesterol was held in 1988 in order to develop a Canadian viewpoint on the association between lipoproteins and atherosclerosis and to develop recommendations and guidelines in this regard. The Canadian Atherosclerosis Society and Department of National Health and Welfare assembled a consensus panel and experts who would present evidence to the panel. The end result was a list of
recommendations for the prevention and treatment of coronary heart disease in Canada. These recommendations include the implementation of health promotion programs to address cardiovascular risk factors, the development of dietary guidelines to reduce population risk as well as the impetus for the agriculture and food industries to produce food to help achieve and maintain lower blood cholesterol levels. With respect to specific lipoprotein goals and risk factors, the recommendations include a population goal of mean total cholesterol levels of 4.9mmol/L (190mg/dL) and the measurement of serum TC, TG, HDL and LDL in individuals with particular priority on those who have known CHD, have a family history of CHD and have several risk factors which places them at risk for developing CHD. The panel also recommends that patients with hypercholesterolemia aim to reduce their total cholesterol to 5.2mmol/L (200mg/dL) or less and detail lifestyle and pharmacological strategies for doing so, depending on the individuals baseline TC and risk factors.

Pursuant to this conference, the Working Group on Hypercholesterolemia and Other Dyslipidemias (WGHOD) was formed by Health Canada in 1995. In May of 2000, based on recent scientific and clinical data, this group published a series of updated recommendations. These recommendations include routine screening of patients with existing CHD, patients with two or more risk factors for developing CHD, those with diabetes mellitus, those with xanthomata, individuals with a family history of CHD as well as men over 40 and women over age 50 to obtain a lipid profile. The group also recommends risk assessment for the development of atherosclerosis. With the patient’s baseline lipid measurements and results of their risk assessment, the group then recommends the determination of target lipid levels. For individuals at high risk an LDL-C target level of 3.0mmol/L is recommended. For those at very high risk, <2.5mmol/L, moderate risk, <4.0mmol/L and low risk individuals are recommended to keep their LDL-C levels below 5.0mmol/L. Finally, with respect to therapeutic strategies for individuals at different levels of risk, the WGHOD recommends that pharmacological lipid-lowering intervention should be started immediately as well as lifestyle changes in those considered to be at high or very high risk for a cardiovascular event. For those at moderate risk, lifestyle changes should be implemented and patients re-evaluated after 3 months. If target lipid levels have not been achieved by this time, drug therapy is recommended. For individuals at low risk of
cardiovascular events, healthy lifestyle changes should be encouraged to achieve target lipid levels. If these levels have still not been achieved by the end of 6 months, drug therapy is recommended. The WGHOD makes it clear in their report that their recommendations are general guidelines and that physicians must evaluate the cost-benefit ratio of lipid-lowering therapy and exercise clinical judgement in each individual case.

1.3. Strategies To Prevent and Treat CHD — The Means

1.3.1. Dietary and Lifestyle Strategies

There are several modifiable risk factors which play a role in the development of CHD. These include hypertension, cigarette smoking, diabetes, obesity, physical activity and an atherogenic diet. The first line of defence in primary and secondary prevention of CHD is usually aimed at these targets. To this end, the NCEP ATP III has developed the therapeutic lifestyle changes (TLC) program as a means of addressing the major modifiable risk factors which contribute to CHD. The primary steps in the TLC program are: 1) reducing dietary intake of saturated fats (<7% of total calories) and cholesterol (<200mg/day), 2) weight reduction, 3) physical activity, 4) increasing dietary intake of viscous (soluble) fiber (10-25g/day). In addition to the TLC program, the panel makes several recommendations regarding the treatment of hypertension and diabetes, smoking prevention and cessation, reduction of obesity, increase in physical activity and dietary modifications. Depending on the number and severity of risk factors (including nonmodifiable risk factors such as age, sex and family history of CHD), lifestyle changes may not be enough to prevent and treat CHD. In these cases, pharmacological strategies are warranted.

1.3.2. Pharmacological Strategies

Several pharmacological strategies have been developed for the prevention and treatment of CHD. The majority of these strategies are aimed at lowering serum total and LDL cholesterol levels as well as increasing serum HDL levels. Some of the major pharmacological agents used for these purposes are outlined here.
1.3.2.1. HMG-CoA Reductase Inhibitors

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, are a large class of well-studied drugs used to treat hypercholesterolemia. De novo endogenous cholesterol synthesis occurs primarily in the liver in a series of enzyme-catalysed reactions beginning with the precursor, acetate. Mevalonate synthesis is the first committed step in the cholesterol synthesis pathway and is carried out by the reduction of 3-hydroxy-3-methylglutaryl CoA by the rate-limiting enzyme HMG-CoA reductase.\(^{43}\)

Inhibition of HMG-CoA reductase is a reversible process requiring only nanomolar concentrations of the drug whereas the concentration of the natural substrate is required in the micromolar range. The potency of HMG-CoA reductase inhibitors is also due, in part, to their three times greater affinity for the enzyme compared to HMG-CoA.\(^{43}\)

In addition to direct inhibition of endogenous cholesterol synthesis, the net lipid-lowering effect of HMG-CoA reductase inhibitors is also due, in part, to the up-regulation of the hepatic LDL-receptor.\(^{44,45}\) When the liver is depleted of cholesterol through the inhibition of endogenous cholesterol synthesis, a compensatory mechanism is set into play which elicits an increase in the expression of LDL-receptor mRNA thereby increasing the amount of cholesterol taken up by the liver.\(^{46}\)

HMG-CoA reductase inhibitors were first discovered in 1976 when Endo and Kuroda isolated a compound from \textit{penicillium citrinum} which lowered cholesterol levels in rats.\(^{47}\) Since this time, many naturally- and synthetically-derived HMG-CoA reductase inhibitors have been developed, intensely studied and widely employed as lipid-lowering agents.

HMG-CoA reductase inhibitors are unique among hypolipidemic drugs in that they directly inhibit endogenous cholesterol synthesis compared to the indirect mechanisms through which other classes of drugs exhibit their lipid-lowering effects.\(^{48}\) The degree to which various HMG-CoA reductase inhibitors exert their effect has been extensively studied in many clinical trials in patients with both primary and secondary hypercholesterolemia.
1.3.2.1.1. WOSCOPS

The West of Scotland Coronary Prevention Study Group set out to determine whether administration of the HMG-CoA reductase inhibitor, pravastatin, to hypercholesterolemic men with no history of myocardial infarction (MI) resulted in reduced incidence of CHD.49 6595 men, aged 45 to 64 with an average serum total cholesterol concentration of 7.0±0.6 mmol/L were randomly assigned to receive 40mg/day pravastatin or placebo. After a mean follow-up period of 4.9 years, they reported that those in the pravastatin group demonstrated a significant 20% reduction in serum total cholesterol and a 26% reduction in serum LDL-C levels. They also reported a 30% reduction in fatal or nonfatal coronary events in the treatment group compared to control with no significant adverse effects.

1.3.2.1.2. AFCAPS/TexCAPS

A more recent primary prevention trial was the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS).50 The aim of this trial was to evaluate the effects of lovastatin (20-40mg/day) treatment on first acute major coronary events in men and women with no prior CHD, average total and LDL-cholesterol levels and below-average HDL-C levels. Results of this study, after an average 5.2 year follow-up period included a 37% reduced incidence of a first acute major coronary event, 25% reduction of LDL cholesterol and a 6% increase in HDL-C. The underlying motivation behind this study was to measure the effects of HMG-CoA reductase inhibitor treatment on the general population as a means of prevention of fatal or nonfatal myocardial infarction, unstable angina or sudden cardiac death. Based on the results, the AFCAPS/TexCAPS research group suggested the possibility of wide-spread, long-term use of low dose HMG-CoA reductase inhibitor treatment for the prevention of CHD.

Evidence for the effects of drug treatment in lowering cholesterol levels and preventing secondary cardiovascular events can be gleaned from several well-recognised trials.
1.3.2.1.3. 4S

The Scandinavian Simvastatin Survival Study (4S) group set out to measure the effects of reduction in serum cholesterol with HMG-CoA reductase inhibitor treatment on overall morbidity and mortality in patients with CHD.\textsuperscript{51} In this trial, 4444 patients, aged 35-70 years, with angina pectoris or a history of MI and a plasma total cholesterol concentration between 5.5-8.0mmol/L were placed on a lipid-lowering diet and randomly assigned to either treatment or placebo. After an average follow-up period of 5.4 years, reductions in total cholesterol (25\%) and LDL-cholesterol (35\%) were shown in the treatment group as well as 8\% increase in mean HDL-cholesterol levels without any corresponding changes in the placebo group. With the primary endpoint being total mortality, they used the Kaplan-Meier 6-year probability of survival and showed that in the treatment group this was increased to 91.3\% from the 87.7\% demonstrated in the placebo. This improvement in survival is accounted for by a 42\% reduction in risk of coronary death.

1.3.2.1.4. CARE

While the 4S study examined the effects of HMG-CoA reductase inhibitors on hypercholesterolemic patients, the Cholesterol and Recurrent Events (CARE) trial sought to determine the effects of HMG-CoA reductase inhibitor treatment in the prevention of secondary cardiac events in patients with average serum cholesterol levels.\textsuperscript{52} Patients were included in the study if they had previous myocardial infarction and had total cholesterol levels less than 6.15mmol/L and LDL-C levels between 2.95 and 4.46mmol/L. 4159 patients were randomly assigned to pravastatin (40mg/day) or placebo and were treated for a median duration of 5 years. The findings of this study were that the primary endpoint of coronary death or recurrent MI was reduced by 24\% on pravastatin treatment and that LDL-C was a significant predictor of coronary event rate.

1.3.2.1.5. LIPID

Another major clinical trial examining the effects of lipid-lowering of HMG-CoA reductase inhibitors in patients without markedly elevated serum cholesterol levels was the Long-Term
Intervention with Pravastatin in Ischaemic Disease (LIPID) study. In this trial, 9014 subjects, aged 31 to 75 with a history of myocardial infarction or unstable angina and plasma cholesterol levels between 4.0 – 7.0mmol/L were given initial dietary counselling and then randomly assigned to treatment with pravastatin, 40mg/day, or placebo. Results of this study were a reported 18% reduction in total cholesterol, 25% reduction in LDL-C, 22% reduction in total mortality and 24% reduction in cardiovascular mortality in the pravastatin group compared to placebo over 6.1 years.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Study Description</th>
<th>Duration (yrs)</th>
<th>LDL-C (%)</th>
<th>Mortality (%)</th>
<th>CAD Death (%)</th>
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<tr>
<td>WOSCOPS</td>
<td>6,595 pts, HC, No CAD Pravastatin 40mg/d</td>
<td>4.9</td>
<td>26</td>
<td>22 (NS)</td>
<td>33</td>
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<tr>
<td>(37)</td>
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<tr>
<td>AFCAPS/TexCAPS</td>
<td>6,605 pts, NC, No CAD Lovastatin 20-40mg/d</td>
<td>5.2</td>
<td>25</td>
<td>NA</td>
<td>36</td>
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<tr>
<td>(38)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4S</td>
<td>4,444 pts, HC, CAD Simvastatin 10-40mg/d</td>
<td>5.0</td>
<td>35</td>
<td>30</td>
<td>42</td>
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<td>(39)</td>
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<tr>
<td>CARE</td>
<td>4,159 pts, NC, CAD Pravastatin 40mg/d</td>
<td>5.0</td>
<td>28</td>
<td>8 (NS)</td>
<td>19</td>
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<td>(40)</td>
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<tr>
<td>LIPID</td>
<td>9,014 pts, NC, CAD Pravastatin 40mg/d</td>
<td>6.1</td>
<td>25</td>
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<td>(41)</td>
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HC=hypercholesterolemia; NC=normocholesterolemia; CAD=coronary artery disease; NS=not significant; NA=not available

Table 2. HMG-CoA reductase inhibitor trials.
Summary of major clinical trials examining the effects of HMG-CoA reductase inhibitors on plasma lipids and coronary events (adapted from Vaughan et al. 2000)

1.3.2.1.6. Safety

HMG-CoA reductase inhibitors have been extensively and effectively used in treating hypercholesterolemia since the advent of lovastatin on the clinical market in 1987. In their fifteen years of use, they have become the most commonly prescribed drugs for lipid-lowering. It wasn’t until August 2001, however, that the safety of routine prescription of HMG-CoA reductase inhibitors was seriously considered. On August 8th, 2001, cerivastatin was voluntarily withdrawn from the US market by its manufacturer due to an abnormally high number of rhabdomyolysis-induced deaths in patients taking cerivastatin and, more
particularly, cerivistatin combined with gemfibrozil. Common side effects of HMG-CoA reductase inhibitors include gastrointestinal disturbances, indigestion, headache, muscle pain, central nervous system disturbances and sleep disorders. On rare occasions, however, one or more forms of muscle toxicity has been reported. Myositis is defined as muscle ache or weakness with creatine kinase (CK) levels up to ten times the normal upper limit. Rhabdomyolysis, on the other hand, is the advent of muscle symptoms with CK levels substantially more than ten times the upper normal limit. This clinical syndrome is caused by injury to the sarcolema of skeletal muscle, subsequent necrosis and the release of the contents of myocytes into the circulatory system. This may then lead to renal obstruction and dysfunction. Myotoxicity, in general, has been reported in 1% to 7% of patients on HMG-CoA reductase inhibitor therapy. From October 1997 to December 2000, 772 cases of nonfatal or fatal rhabdomyolysis from use of all HMG-CoA reductase inhibitors were reported with 73 deaths from this cause of the 480 000 000 prescriptions dispensed. The rate of fatal rhabdomyolysis was 16 to 80 times greater with cerivistatin than for that of any other HMG-CoA reductase inhibitor. While cerivistatin has been withdrawn from the market, there have still been documented cases of fatal rhabdomyolysis with other HMG-CoA reductase inhibitors.

Another complication arising from HMG-CoA reductase inhibitor therapy is liver toxicity including hepatitis and increased levels of hepatic transaminase concentrations. Dose-dependent elevated levels have been reported in 0.5% to 2.0% of cases and it is speculated that this may lead to hepatotoxicity.

People at highest risk for experiencing adverse events while on HMG-CoA reductase inhibitor therapy are the elderly (especially over 80 years), those with active liver or muscle disease, patients with untreated hypo- or hyperthyroidism, those on immunosuppressive medication and women of childbearing potential due to risk of fetal malformation. Certain drug interactions with HMG-CoA reductase inhibitors should also be carefully monitored especially fibrates, erythromycin, itraconazole and immunosuppressive drugs such as cyclosporin which may lead to increased circulatory levels of HMG-CoA reductase inhibitors and therefore increase the risk for myopathies.
Having examined all of these potential adverse events with HMG-CoA reductase inhibitor therapy, it is important to note that severe side-effects are very rare and are often reversed if HMG-CoA reductase inhibitor therapy is discontinued. In addition, the benefits offered by HMG-CoA reductase inhibitors in reducing CHD-associated morbidity and mortality outweigh the mild risks associated with HMG-CoA reductase inhibitor therapy. Nonetheless, there are some very real concerns especially with the prolonged use of high-dose HMG-CoA reductase inhibitors.

1.3.2.2. Bile Acid Sequestrants

Bile acid sequestrants, also called bile acid binding resins, are another class of lipid-lowering drugs. These drugs function by binding to bile acids in the small intestine and forming an insoluble complex which is excreted in the feces. The net result of this action is an interruption of the enterohepatic circulation of bile acids. This, in turn, disrupts hepatic cholesterol homeostasis and leads to increased expression of the LDL-receptor thereby increasing LDL catabolism and decreasing plasma LDL-C levels. In the Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT), hypercholesterolemic men were treated with the bile acid resin cholestyramine. After a 7-year follow-up period, a significant relationship was demonstrated between cholestyramine consumption, total and LDL-cholesterol lowering and reduction in CHD risk. Specifically, a mean reduction of plasma TC of 8%, of plasma LDL-C of 12% and of incidence of CHD of 19% were reported. The major drawbacks to bile acid sequestrant therapy is their bulkiness and inconvenience of administration. The two most commonly-used resins, cholestyramine and cholestipol are administered as powders that must be dissolved in water or juice and taken twice daily. These resins are also not ideal because of their side effects which include a wide array of gastrointestinal symptoms. A new, more potent, bile acid binding resin has recently been marketed. Colesevelam is more easily-administered than traditional resins and is well tolerated with patients displaying a marked reduction in gastrointestinal side-effects.
1.3.2.3. Nicotinic Acid

Nicotinic acid is a hydrophilic B-complex vitamin that has been used to effectively lower LDL-cholesterol and triglyceride levels as well as to increase serum HDL-C. Nicotinic acid has also been shown to transform the more atherogenic small, dense LDL into larger, more buoyant particles. It seems to function by inhibiting hepatic VLDL synthesis and secretion through the inhibition of peripheral mobilisation of free fatty acids. When lipolysis is decreased in adipose tissue, there is a corresponding decrease in plasma free fatty-acids—the main precursors of VLDL-triglycerides. The decrease in VLDL synthesis, then, leads to a corresponding decrease in IDL and LDL particles. In the Coronary Drug Project, a major clinical trial using niacin monotherapy, the administration of 1-3g daily resulted in a 10% and 26% decrease in plasma cholesterol and triglycerides, respectively. This resulted in a 27% decrease in nonfatal myocardial infarction. Several angiographic trials have also demonstrated the benefits of nicotinic acid in reducing the progression of atherosclerosis. Despite its benefits, however, niacin therapy is accompanied by a plethora of potential side-effects including gastrointestinal symptoms, hepatotoxicity, gout and hyperglycemia with reduced insulin sensitivity. Even with these side-effects, niacin therapy has been shown to be an effective pharmacological choice in patients with severe combined hyperlipidemia due to its concomitant effect of reducing both LDL-C and triglycerides.

1.3.2.4. Fibrates

Fibric acid derivatives are another class of drugs that have been used to treat various forms of dyslipidemia particularly those involving hypertriglyceridemia. In various clinical trials, fibrates have been shown to effectively lower plasma cholesterol and triglyceride levels while raising HDL-C levels. They have also been shown to decrease the incidence of nonfatal myocardial infarction. The fibrate, gemfibrozil, was used in the Helsinki Heart Study to examine its effect on primary prevention of coronary events in middle-aged, dyslipidemic men. This trial reported a positive association between lowering of total (8%) and LDL-cholesterol (8%) as well as increasing plasma HDL-cholesterol (10%) with reduction (34%) in incidence of CHD. Fibrates act through an intricate mechanism in which they play a role in activating peroxisome proliferator-activated receptors, particularly the
alpha form (PPAR-α) which is predominantly expressed in the liver. This is thought to result in the increased expression of lipoprotein lipase and the decreased expression apolipoprotein C-III (an inhibitor of lipoprotein lipase). This also results in increased expression of genes for apolipoprotein A-I and -II, fatty acid oxidation and fatty acid transport protein. The simultaneous increase in lipoprotein lipase and decrease in apo C-III is primarily responsible for the hypotriglyceridemic effects seen with fibrate therapy. This hypolipidemic effect is further compounded by an induction of hepatic fatty-acid uptake by fatty acid transport protein alongside a reduction of hepatic triglyceride production which leads to a reduced availability of fatty acids for triglyceride synthesis in the liver. A reduction in plasma LDL levels is also seen with fibrate therapy. This is a result of decreased triglyceride availability which leads to the formation of larger, less dense LDL which, in turn, have a greater affinity for the LDL receptor thereby increasing the number of LDL particles removed from the plasma. Finally, fibrates have also been shown to increase concentrations of plasma HDL. This is due to the increased expression of apo A-I and A-II which facilitate reverse cholesterol transport. Although fibrates are generally well-tolerated, some side effects may include gastrointestinal symptoms, increased incidence of gallstones and increased risk of myopathy when combined with HMG-CoA reductase inhibitor therapy.

1.3.2.5. Cholesterol Absorption Inhibitors

A recent addition to the cholesterol-lowering market is the cholesterol absorption inhibitor, ezetimibe. Ezetimibe functions by inhibiting the absorption of dietary and biliary cholesterol across the intestinal wall. At the same time, ezetimibe does not affect the absorption of fatty acids, fat-soluble vitamins or triglycerides. Because this drug is so new, only smaller-sized, shorter duration clinical trials have been conducted. The results of these trials, however, have demonstrated that ezetimibe, administered once daily in a 10mg dose to patients with hypercholesterolemia, reduces plasma total- and LDL-cholesterol, apolipoprotein B and triglycerides while increasing serum HDL-C. Specifically, two studies of ezetimibe 10mg/day in 1,719 hypercholesterolemic subjects resulted in an 19.1% average reduction of LDL-C compared to placebo. In all studies with ezetimibe, it has been shown to be well-tolerated with no difference in side-effects from those reported in
Although little is known about the molecular target of ezetimibe, its effectiveness lies in its long half-life and its survival of enterohepatic circulation. This allows the drug to be returned to its primary site of action and limits its exposure to peripheral tissues. Because ezetimibe only targets the exogenous cholesterol pathway, homeostatic mechanisms to increase endogenous cholesterol synthesis have been shown to come into play with ezetimibe treatment. For this reason, ezetimibe therapy is most effective when combined with HMG-CoA reductase inhibitor therapy in order to target both the endogenous and exogenous cholesterol pathways simultaneously. In this respect, several short-term trials with HMG-CoA reductase inhibitors have shown to further reduce LDL-C levels, most notably in patients who had been unable to achieve the ATP II goals on HMG-CoA reductase inhibitor monotherapy.

1.3.2.6. Phytosterols

Dietary sources of plant sterols, or phytosterols, have been used since the 1950s to treat hypercholesterolemia. These compounds which are structurally similar to cholesterol, differing only in the C-24 position of the side chain, assumedly inhibit cholesterol absorption by displacing cholesterol from the mixed micelle in the small intestine. This displacement occurs through plants sterols decreasing the solubility of cholesterol in the oil and micellar phases thereby interfering with the absorption of cholesterol. Unlike cholesterol, however, plant sterols are only absorbed to a small extent due to the additional side chain at C-24 of cholesterol – the longer the side chain, the less the phytosterol is absorbed due to its increased hydrophobicity. Phytostanols, the saturated counterparts of phytosterols, are even more hydrophobic and are therefore virtually unabsorbed. Additionally, phytostanols remain in the intestinal lumen and continuously interfere with solubility of cholesterol in the micelle.

The first study conducted in humans using phytosterols as hypolipidemic agents, employed sitosterol in a powdered form. Administering 5-7g/day of sitosterol to 26 hypercholesterolemic men, Pollak demonstrated that a 28% reduction in serum total cholesterol was achieved compared to control. In the 1970s, further studies were published reporting the use of smaller doses of plant sterols. In these studies, 3-6g/day of...
Phytosterol administration resulted in reductions of plasma total cholesterol of about 12%. Notably, the 3g dose achieved the highest reduction of LDL-C whereas increasing the dose to 6g had no further effect on serum cholesterol levels. Further studies in humans were conducted using plant stanols which had been shown to be even more effective than plant sterols at inhibiting cholesterol absorption in animal studies. When 1.5g/day of sitostanol was administered to 6 hypercholesterolemic patients over the course of 4 weeks, there was a reported 15% reduction in total cholesterol due entirely to a decrease in LDL cholesterol.

Because of inconsistencies in results reported when phytostanols were administered in different forms, Finnish investigators developed a fat-soluble form of plant stanols wherein plant stanols are esterified to fatty acids thereby rendering them soluble in fat-based foods. In a randomised, double-blind study, 153 patients with mild hypercholesterolemia substituted part of their daily fat intake with margarine containing sitostanol ester or placebo. At the end of the year-long treatment period, there was a mean reduction in total cholesterol and LDL-cholesterol in the sitostanol group of 10.2% and 14.1% respectively compared to an increase in total cholesterol of 0.1% and a decrease in LDL-C of 1.1% in the control group.

The mechanism through which phytosterols lower serum total- and LDL-cholesterol is very similar to that employed by other cholesterol absorption inhibitors. When phytosterols displace cholesterol from the mixed micelle and thereby prevent cholesterol from being absorbed, there is a reduced concentration of cholesterol being incorporated into the chylomicrons secreted into the bloodstream, taken up by the liver and incorporated into VLDL. This decrease then translates into a reduction in plasma LDL. Reduction in plasma LDL-C levels, in turn, leads to increased hepatic endogenous cholesterol synthesis as well as increased expression of the LDL receptor in the liver which subsequently results in greater uptake of LDL from the blood thereby further decreasing plasma LDL-C.

Although the effect of plant sterols on atherogenesis in humans has not been tested, several studies in animal models have demonstrated decreased atherosclorotic plaque formation secondary to treatment with dietary phytosterols. In the rabbit model, Ikea et al. demonstrated a reduction in aortic atheroma in animals fed 0.5% (wt/wt) cholesterol and 0.5% (wt/wt) β-sitostanol compared to those fed 0.5% (wt/wt) cholesterol alone.
comparing the effects of \( \beta \)-sitostanol to \( \beta \)-sitosterol on atherosclerotic lesion formation, they demonstrated that \( \beta \)-sitostanol was more effective at both reducing plasma total cholesterol as well as reducing atherosclerotic lesion area.\(^8\) Similarly, in rabbits fed 0.5% cholesterol diets and graded amounts of sitostanol (0.01, 0.2 and 0.8% (wt/wt)), atherosclerotic plaque development was significantly reduced, and almost entirely prevented, in animals fed 0.8% (wt/wt) sitosterol compared to control.\(^9\) Studies in apolipoprotein E-deficient mice have demonstrated similar findings. When apo E-deficient mice on a 0.15% (wt/wt) cholesterol diet were fed a 2% (wt/wt) tall oil-derived phytostanol mixture, a significant reduction in plasma total cholesterol was demonstrated in treated mice compared to control animals.\(^9\) Additionally, atherosclerotic lesion area was reduced by more than half in the treated animals compared to control.

The evidence from animal studies and clinical trials discussed here presents a strong case for the employment of phytosterols in lipid-lowering and preventing atherosclerosis. Because of their efficacy and safety profile with virtually no side-effects when administered in small quantities, some even suggest that stanol esters should be used in long-term feeding in mass intervention studies.\(^8\)
Figure 1. Sites of Action of Some Major Lipid-Lowering Drugs.

CE = cholesteryl esters; FC = Free cholesterol; ACAT = acyl-CoA:cholesterol acyltransferase
1.4. **FURTHER LIPID-LOWERING — THE FOCUS**

It is abundantly clear from the evidence presented that elevated plasma cholesterol levels pose an increased risk for atherosclerosis and that reducing plasma cholesterol levels, especially in the LDL, fraction leads to a reduction in risk for coronary heart disease. It is also clear that there are many available pharmacological strategies for achieving lower cholesterol levels among which HMG-CoA reductase inhibitors are currently the most widely used. Target plasma total- and LDL-cholesterol levels, as set out by various guidelines (see NCEP ATP III and CCCC above) are often not met on HMG-CoA reductase inhibitor monotherapy. In addition, angiographic trials have shown that an approximate 45% reduction of LDL-C is necessary to lead to an actual regression of atherosclerosis. In the major HMG-CoA reductase inhibitor trials, the highest degree of LDL-C reduction was seen in the 4S study where a 35% reduction in LDL-C was achieved in hypercholesterolemic patients whereas there was only a 25% reduction in LDL-C in the AFCAPS/TexCAPS and LIPID studies. While these results are impressive, the LDL-C levels achieved are still well above the baseline levels of countries where CHD is rare. In addition, the Lipid Treatment Assessment Project (L-TAP) demonstrated that, in dyslipidemic adults receiving hypolipidemic therapy for at least 3 months, only 38% of patients achieved LDL-C levels as set out by the National Cholesterol Education Program. For these reasons, more aggressive lipid-lowering is needed.

More aggressive therapy with HMG-CoA reductase inhibitors, however, may not be entirely warranted. For every doubling of HMG-CoA reductase inhibitor dose, there is generally only a 5-6% decrease in LDL-C levels. This then further raises the question of the safety of long-term use of high doses of HMG-CoA reductase inhibitors. Instead of merely increasing HMG-CoA reductase inhibitor doses, adjunct therapy is prudent to assist in lowering the LDL-C burden and assist patients in achieving greater reductions in serum cholesterol levels and thus decreasing the burden of CHD.
2. CONTEXT AND FRAMEWORK FOR THE CURRENT STUDY

2.1. FM-VP4

2.1.1. Chemistry of FM-VP4

Disodium Ascorbyl Phytostanol Phosphates (FM-VP4) is a novel, water-soluble phytostanol analog that inhibits cholesterol absorption. FM-VP4 has 2 major components: disodium ascorbyl campestaneryl phosphate and disodium ascorbyl sitostanyl phosphates (Figure 2). FM-VP4 is produced by the catalytic hydrogenation of campesterol and sitosterol to form campetstanol and sitostanol which are then each linked to ascorbic acid by a phosphodiester bond.\(^9\) FM-VP4 has an average formula weight of 693.65 g/mol.

![Figure 2. Chemical structure of disodium ascorbyl phytostanol phosphates (FM-VP4).](image)

2.1.2. Animals Studies with FM-VP4

2.1.2.1. Rats

In order to determine the plasma pharmacokinetics of cholesterol following FM-VP4 administration, adult male Sprague Dawley rats were administered single oral doses of 254mCi/ml [3H] cholesterol alone or with 5, 10, 20, 50 or 100mg/kg FM-VP4.\(^9\) This
treatment resulted in a significant, dose-dependent decrease in the \([^3\text{H}]-\text{cholesterol AUC}_{0-48\text{h}}\) (total body exposure of cholesterol during the 48-hour period) and \(C_{\text{max}}\) (the maximum concentration of cholesterol in systemic circulation). Co-administration of tritiated cholesterol with 10, 20, 50 and 100mg/kg also resulted in significant increases in CL/F (the rate of cholesterol removal from systemic circulation) and Vd/F (total body distribution of cholesterol).

In order to determine the pharmacokinetics, tissue distribution and excretion of FM-VP4, rats were administered a single oral (150mg/kg) or intravenous (15mg/kg) dose of radiolabelled FM-VP4 and urine, feces and blood were collected at various time points between 0 and 48 hours. At the end of the 48-hour period, animals were sacrificed and several tissues were harvested. Results of tissue and sample analyses revealed that the majority of the \[^3\text{H}]\text{FM-VP4}\) was recovered in the feces and gastrointestinal tract and that FM-VP4 displayed an oral bioavailability of only 6.5%.

2.1.2.2. Gerbils

Mongolian gerbils are LDL-dominant animals which exhibit sensitivity to dietary cholesterol. In a 4-week study conducted in this model to determine the plasma lipoprotein response to acute oral administration of FM-VP4, significant decreases in total plasma cholesterol and LDL-cholesterol levels were demonstrated. Animals were fed a diet containing either 0, 0.25, 0.5, 1.0 or 2% (wt/wt) FM-VP4 mixed in standard gerbil chow \((n=6\) in each group). After the 4-week treatment period, the blood plasma was analysed for concentration of lipid constituents. Significant decreases were demonstrated in both total- and LDL-cholesterol fractions compared to control when animals received either 1% or 2% (wt/wt) FM-VP4 in their diet. Additionally, a significant reduction in weight was observed after the 4-week period in those animals consuming 1% or 2% (wt/wt) dietary FM-VP4 compared to controls. No significant change in HDL-C or triglycerides, however, was observed.

A second study was conducted in gerbils to determine the dose-response effect of chronic oral administration of FM-VP4 in this model. In this study, animals were given 2% or 4%
FM-VP4 incorporated into standard gerbil chow or 2% or 4% (wt/vol) FM-VP4 dissolved in drinking water (n=6 in each group). At the end of the 8-week study period, blood was analysed for total cholesterol, total triglycerides, HDL-C and LDL-C as well as for aspartate amino transferase (AST), alanine amino transferase (ALT) and creatinine levels. Results demonstrated a significant decrease in both plasma total- and LDL-cholesterol levels compared to controls in animals receiving 2% or 4% (wt/wt) or (wt/vol) FM-VP4 in either food or water. Animals on the 4% (wt/wt) or (wt/vol) FM-VP4 treatment (in either food or water) also demonstrated a significant reduction in body weight compared to controls while no differences in food or water intake were measured. Triglycerides were shown to be significantly decreased in animals receiving 4% (wt/vol) FM-VP4 in water compared to controls whereas a significant increase in HDL-cholesterol levels was demonstrated in animals administered either 2% (wt/wt) FM-VP4 in food or 4% (wt/vol) in water. No significant change in plasma aspartate amino transferase (AST), alanine amino transferase (ALT) or creatinine levels were observed in any of the animals receiving FM-VP4 treatment suggesting that FM-VP4 had no averse affect on liver, heart or renal function.

2.1.2.3. Apolipoprotein E-Deficient Mice

Apo E-deficient mice have been shown to be hyper-responsive to dietary cholesterol, exhibiting delayed clearance of plasma lipoproteins, overwhelming concentrations of plasma cholesterol and accelerated atherogenesis. These mice were fed a diet containing 0.2% (wt/wt) cholesterol and 9.0% (wt/wt) fat and treated with either 0.1%, 0.5%, 1.0% or 2% (wt/vol) FM-VP4 in their drinking water or 2% (wt/wt) FM-VP4 in their diet. After 12 weeks mice receiving 0.5%, 1% and 2% (wt/wt) or (wt/vol) FM-VP4 exhibited a significant (approximately 75%) reduction in plasma total cholesterol levels. In those mice receiving 2% (wt/wt) or (wt/vol) FM-VP4, a significant reduction in plasma triglycerides was shown at weeks 4 and 8 compared to controls. Atherosclerotic lesion analysis revealed a 75% reduction in lesion area in those mice receiving 0.5%, 0.1% or 2.0% (wt/wt) or (wt/vol) FM-VP4 compared to controls. Comparison between mice receiving either or both of FM-VP4’s parent compounds (ascorbic acid and FM-3P4: parent phytostanols) demonstrated that the net effect of FM-VP4 on serum lipoprotein and atherosclerotic lesion formation was more potent than that of either or both of its parent compounds administered under the
same conditions. Additionally, while there was no change in plasma cholesterol or triglyceride levels in animals receiving 0.1% FM-VP4, there was, nonetheless, a significant reduction in lesion area observed. This suggests that FM-VP4 may have an antiatherosclerotic effect independent of its effect on serum lipids.
3. HYPOTHESES

a) FM-VP4 lowers serum total and LDL cholesterol levels in a dose-response manner and leads to a subsequent reduction in atherosclerotic lesion formation.

b) FM-VP4 exerts its cholesterol-lowering effect by increasing the expression of the ABCA1, ABCG5, ABCG8 and LDL receptor genes.

4. SPECIFICAIMS

Aim 1: To measure the dose-response effect of oral administration of FM-VP4 on serum lipids in the hypercholesterolemic rabbit model.

Aim 2: To measure the effects of oral administration of FM-VP4 on cholesterol-lowering and atherosclerotic lesion formation in the cholesterol-fed apo B-100 transgenic mouse.

Aim 3: To measure the relative expression of the ABCA1, ABCG5, ABCG8 and LDL receptor genes in the liver and small intestine in response to FM-VP4 treatment in the cholesterol-fed apo B-100 transgenic mouse.
5. **RATIONALE**

5.1. **THE HYPERCHOLESTEROLEMIC RABBIT MODEL**

Findings from our laboratory demonstrate that FM-VP4 inhibits cholesterol absorption in several animal models and leads to a subsequent reduction in plasma total cholesterol, LDL-cholesterol and atherosclerotic lesion area. Rabbits are an appropriate model for the study of the effects of lipid-lowering agents since serum cholesterol levels can be manipulated by dietary means to induce hypercholesterolaemia.\(^{95-97}\) Additionally, it is well established that oral administration of phytostanols to cholesterol-fed rabbits results in a reduction in total cholesterol, LDL-cholesterol and extent of atherogenesis.\(^{83,84,98}\) The cholesterol-fed rabbit model has also been used to demonstrate the lipid-lowering effects of HMG-CoA reductase inhibitors.\(^{99}\) It has been shown that plant sterol-induced inhibition of cholesterol absorption is associated with increased endogenous cholesterol synthesis.\(^{100,101}\) Because of the safety concerns associated with prolonged use of high doses of HMG-CoA reductase inhibitors,\(^{54,58}\) the fact that some patients are poor responders to HMG-CoA reductase therapy,\(^{102}\) and the evidence that target LDL-cholesterol levels are not being achieved with HMG-CoA reductase inhibitor therapy alone,\(^{57}\) we sought to develop a hypercholesterolemic animal model in which the lipid-lowering and atheroprotective effects of FM-VP4 could be tested with the concomitant administration of other lipid-lowering agents.

5.2. **THE APO B-100 TRANSGENIC MOUSE MODEL**

The mouse is a widely used animal model because of its size, cost and ease of handling. Wild-type mice, however are highly resistant to atherosclerosis, have relatively low levels of plasma cholesterol and carry their cholesterol in HDL.\(^{93}\) Apo E-deficient mice display very high concentrations of plasma cholesterol (10-15mmol/L) which is present primarily in VLDL.\(^{18,103}\) While apo E-deficient mice develop extensive atherosclerotic lesions similar in appearance and distribution to human lesions, their lipoprotein profile is quite different.\(^{19,93}\) Transgenic mice expressing the human apo B gene, however, display an LDL-dominant lipoprotein profile that more closely resembles the human plasma lipoprotein distribution.\(^{104}\) These mice have elevated levels of LDL cholesterol even on a regular chow diet.\(^{105}\) When
fed a diet rich in cholesterol and fat, this strain of transgenic mice has demonstrated significantly increased levels of total and non-HDL cholesterol compared to nontransgenic controls on the same diet. Additionally, apo B-100 transgenic mice on this atherogenic diet have been shown to develop extensive lipid-rich atherosclerotic lesions. For these reasons, we chose the apo B-100 transgenic mouse model to further study the lipid-lowering and atheroprotective effects of FM-VP4 in a mouse model that more closely resembles the human hyperlipidemic and atherogenic state.

We also sought to use the apo B-100 transgenic mouse model to investigate the molecular mechanism of action through which FM-VP4 functions by measuring the expression of various lipid-regulating proteins.

5.2.1. ABC Transporters

The ATP-binding cassette (ABC) transporter gene family is a large class of membrane proteins which function in the transport of a wide variety of substrates across the cell membrane. ABCA1 serves to transport cholesterol and phospholipids to apolipoproteins bound to the cell surface. ABCA1 seems to target pools of excess cellular cholesterol for transport out of the cell. Fulfilling this function, ABCA1 mediates the first step in reverse cholesterol transport which functions to remove cholesterol from peripheral tissues and deliver it in HDL to the liver for elimination from the body. This places ABCA1 in a key role for facilitating the prevention of atherogenesis. Studies have indeed shown that overexpression of ABCA1 protects against atherosclerosis in apo E-deficient mice which spontaneously develop atherosclerosis. Studies have also shown that people with defective ABCA1 and consequent HDL deficiency (Tangier disease) develop severe hypercholesterolemia and CHD. In examining the effects of plant stanol administration to Caco-2 cells (a cell model of intestinal lipoprotein metabolism), Plat and Mensink demonstrated a significant induction of ABCA1-mediated cholesterol efflux subsequent to sitostanol treatment in the cells compared to control. These findings led us to believe that ABCA1 may play a role in mechanism through which FM-VP4 inhibits cholesterol absorption.
ABCG5 and ABCG8 are also involved in sterol transport. In sitosterolemia, an autosomal recessive disorder, patients demonstrate increased intestinal absorption and decreased biliary secretion of dietary sterols.\textsuperscript{111} Patients with this disorder present with hypercholesterolemia and develop premature coronary atherosclerosis. While normal humans only absorb approximately 5% of dietary phytosterols, most of which is then secreted in the bile, patients with sitosterolemia absorb between 15 and 60% of plant sterols they consume and secrete little of it in the bile.\textsuperscript{111} Several mutations in the genes encoding for ABCG5 and ABCG8 have been identified in patients with sitosterolemia. ABCG5 and ABCG8 function in the export of absorbed cholesterol and phytosterols out of the enterocyte and back into the intestinal lumen.\textsuperscript{112} Because of their involvement in cholesterol and phytosterol transport out of the enterocyte thereby limiting cholesterol absorption, ABCG5 and ABCG8 expression may be affected by FM-VP4 administration.

5.2.2. LDL Receptor

The low density lipoprotein receptor functions in the removal of LDL and VLDL from the plasma for degradation in the liver. People with a mutation in the gene for this receptor develop severe hypercholesterolemia and premature atherosclerosis. It was studies in people with this genetic disorder, familial hypercholesterolemia (FH), that first led to the elucidation of this receptor.\textsuperscript{31} Studies in patients with FH demonstrated that the LDL receptor plays a key role in cholesterol homeostasis. When plasma cholesterol levels are high and cellular cholesterol pools are depleted, there is an increased expression of the LDL receptor. When the demand for cholesterol is less, LDL receptor expression is downregulated to accommodate the decreased need for cellular cholesterol. It has been reported that LDL receptor expression is increased in the mononuclear blood cells of subjects receiving treatment with dietary phytostanols.\textsuperscript{101} These data led us to seek to identify the role that FM-VP4 treatment plays in the regulation of the expression of the LDL receptor.
6. MATERIALS AND METHODS

6.1. HYPERCHOLESTEROLEMIC RABBIT STUDY

6.1.1. Animals

20 female New Zealand White (NZW) rabbits weighing approximately 3.0kg were purchased from Charles River Laboratories (Wilmington, MA) and acclimatized for 7 days. On day 7, 3ml of blood was drawn from the central ear artery for the determination of baseline plasma lipids. During a further 4-week period, rabbits were daily fed bread cubes of different bread types in order to train them to eat this vehicle and to determine their preference of bread type. By the end of this 4-week period, rabbits who would not comply with this feeding were excluded from the study. Rabbits were housed individually in a room with a constant temperature (19-23°C) and humidity (45-65%) and a 12-hour light/dark cycle. During the acclimatization and bread-feeding period, rabbits were fed regular chow (Manna Pro 16% protein) and tap water ad libitum.

6.1.2. Test Substance

FM-VP4 was obtained from BRI Pharmaceutical Research Inc. (BRI ID: FM-VP4-04 lot# 2670-AL-3P). FM-VP4 was suspended in distilled water at a concentration of 20% wt/vol by sonicator.

6.1.3. Drug Administration

In order to determine the dose-response relationship of FM-VP4 to lipid-lowering, it is important that a known quantity of drug is administered. Because of the invasive nature of oral gavage, we used bread cubes as a drug vehicle for the daily administration of FM-VP4. This method was previously use by Kroon et al. to administer mevinolin (lovastatin) to rabbits. The test substance was pipetted on to Silver Hills 16 Grain bread cubes measuring approximately 1.5cm². Different amounts were pipetted onto different cubes depending on the treatment group to which they would be subsequently fed such that each
animal received 1 cube of bread per kilogram of body weight (animals in highest dose group received 2 cubes per kilogram of body weight in order to accommodate the volume of liquid that needed to be added in order to ultimately yield a dose of 100mg/kg). After the application of the test substance, cubes were allowed to dry overnight in semi-covered containers or were dried for 3-4 hours at 60°C in a dry incubator. Presence of FM-VP4 on bread was verified by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). This method first uses liquid chromatography to resolve the different components of a mixture. Each component is then analyzed, based on its chemical bond fragmentation, to confirm the presence of various compounds compared to control samples by mass spectrometry. The second mass spectrometry is used to analyze the subcomponents of each component within the original mixture. Most of the rabbits ate all of their bread cubes within 2 minutes of their having been fed.

6.1.4. Induction of Hypercholesterolemia

Induction of hypercholesterolemia in the rabbit by dietary means is well-established. Different studies, however, have used different concentrations of dietary cholesterol and achieved different degrees of hypercholesterolemia. We originally chose a dietary cholesterol concentration of 0.5% (wt/wt) since this concentration was used by Ikeda et al. in their study of the effect of the antihypercholesterolemic effect of dietary sitostanol in rabbits as well as by Ntanios et al. in their study of the effect of dietary sitostanol on atherosclerotic plaque formation in rabbits.

Subsequent to the 4-week bread-feeding period, animals were placed on a 0.5% (wt/wt) cholesterol diet purchased from Harlan Teklad. After the first week of feeding, animals were weighed and bled from the central ear artery and their plasma total cholesterol (TC) levels were used as a basis for assignment of 3 animals into each of 5 treatment groups: control, 1, 5, 20 and 50mg FM-VP4/kg body weight. At the end of 3 weeks, cholesterol feeding was suspended due to overwhelmingly high plasma TC levels (17-41mmol/L) in all groups and signs of illness in some animals (lethargy & corneal arcus). Animals were subsequently subjected to a wash-out period during which time the cholesterol diet was replaced with regular rabbit chow.
In order to determine a more suitable concentration of dietary cholesterol for the development of mild hypercholesterolemia, animals who had been previously excluded from the study after only 1 week of cholesterol feeding were washed out on regular rabbit chow for 5 weeks. These animals were subsequently fed a 0.05% (wt/wt) CH diet. This diet was made by mixing regular rabbit pellets with 0.5% CH rabbit pellets. After 2 weeks on the 0.05% (wt/wt) CH diet, plasma TC levels had still not increased significantly compared to the new baseline therefore the cholesterol content of the diet was increased to 0.1% (wt/wt) mixed in regular chow. After only one week on the 0.1% (wt/wt) CH diet, plasma TC levels in all of the animals had increased significantly compared to TC levels after 2 weeks on the 0.05% (wt/wt) CH diet.

Meanwhile, animals from the original treatment groups were washed out for 8 weeks and subsequently placed on the 0.1% (wt/wt) CH diet (200g/animal/day). After one week of cholesterol feeding, animals were separated into new treatment groups based on their new baseline TC. The new groups were: control, 2:1 (25mg/kg), 1:1 (50mg/kg) and 1:2 (100mg/kg).

6.1.5. Drug Dose

The initial doses of drug chosen were 1, 5, 20 and 50mg FM-VP4/kg of body weight. These doses were originally chosen in order to examine the effect of the drug through a great range of concentrations with the lower concentrations also having the potential of being translated into dose-equivalents for human therapy with FM-VP4. After the washout period and the subsequent re-entry into the experiment with a diet containing 0.1% (wt/wt) cholesterol, drug doses were also modified. Because FM-VP4 appears to function through competitive inhibition of cholesterol incorporation into the mixed micelle, we chose FM-VP4 doses that were similar in concentration, on a weight-to-weight basis, to the concentration of cholesterol being consumed. Based on the average consumption of approximately 200g per day of food by rabbits and an average body weight of 4kg, we worked out the following ratios of cholesterol to FM-VP4 (CH:FM-VP4) consumption: 2:1 (25mg/kg FM-VP4), 1:1 (50mg/kg FM-VP4) and 1:2 (100mg/kg FM-VP4).
6.1.6. Study Design

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Table 3. Study design.
The identity of treatment groups and timeline for the study of the dose-response relationship of FM-VP4 in the hypercholesteremic rabbit model.

Prior to commencing drug treatment, rabbits were placed on the cholesterol diet alone for one week. The purpose of this cholesterol run-in period was to establish mild hypercholesterolemia prior to drug administration in order to simulate the human condition of seeking drug therapy in response to high lipid levels. At the end of each week, for the first 4 weeks, body weight was measured and blood was drawn for plasma lipid measurement. Due to the negligible effect being observed between each week, it was decided to draw blood and measure lipids every two weeks for the last six weeks of the study.

At the end of the treatment period, animals were euthanized and sections of liver and intestine were removed, flash frozen in liquid nitrogen and stored at -80°C for future analysis.

6.1.7. Blood Sampling

Each rabbit was sedated by intramuscular injection of 0.1ml Tobugisic & 0.1 ml Atravet following an 8-hour fast. After an approximately 5-minute waiting period, the outside of one ear was shaved and cleaned with rubbing alcohol. Blood was drawn using a 21-guage
catheter into 3ml EDTA-coated vacutainer tubes. Plasma was separated by centrifugation at 3000rpm for 10 minutes and stored in cryogenic tubes at -20°C until analyzed.

6.1.8. Lipid Analysis

Plasma total cholesterol (TC) and triglyceride (TG) levels were quantified using enzymatic kits (Sigma Aldrich). Both kits rely on colorometric assays in which the absorbance of the final product is measured at a specified wavelength in a spectrophotometer. The first step in the cholesterol assay is the enzymatic hydrolysis of esterified cholesterol by cholesterol esterase to yield free cholesterol and fatty acids. Free cholesterol – both that originally present in the plasma and that which has just been hydrolyzed, is oxidized by cholesterol oxidase to give cholest-4-en-3-one and hydrogen peroxide. The third and final step involved the reaction of hydrogen peroxide with hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase to form a quinoneimine dye which is then spectrophotometrically quantified, compared to a blank, at 500-550nm.

The triglyceride assay follows similar principles. In this assay, the first step is the enzymatic hydrolysis of triglycerides by lipase to yield glycerol and free fatty acids. Next, the glycerol is phosphorylated by adenosine triphosphate with glycerol kinase thereby producing glycerol-3-phosphate and adenosine diphosphate. Thirdly, glycerol phosphate oxidase acts on glycerol-3-phosphate to yield dihydroxyacetone phosphate and hydrogen peroxide. Finally, peroxidase catalyses the final reaction of hydrogen peroxide with 4-aminoantipyrine and 3,5 dichloro-2-hydroxybenzene sulfonate to produce a red-coloured dye whose absorbance at 500nm is proportional to the concentration of triglycerides in the sample.

HDL was measured using the dextran sulfate-Mg$^{2+}$ method per Warnick et al. wherein apolipoprotein B-containing lipoproteins are precipitated out of the plasma sample following the addition of dextran sulfate and magnesium chloride.$^{116}$ The cholesterol remaining in the supernatant, once the precipitate in pelleted by centrifugation, is, assumedly, contained in the HDL fraction as is then measured by the enzymatic reaction described above.
LDL levels were calculated using the Friedewald equation.\textsuperscript{117}

$$LDL-C = TC - HDL-C - TG/5$$

This equation assumes that almost all total plasma cholesterol is distributed in the three major lipoprotein classes: VLDL, LDL and HDL.\textsuperscript{118} This equation also assumes that the ratio of triglycerides:cholesterol in normal cholesterol is about 5:1. For this reason, this equation should not be used in samples with very high triglycerides or a high plasma concentration of chylomicrons (i.e. non-fasting samples).\textsuperscript{118}

6.1.9. Statistical Analysis

Continuous factors were compared using two-tailed student's t-test with unequal variance. Data are presented as means ± standard deviations. Values were considered significant if p<0.05. All data were analysed using the basic statistical functions provided in Microsoft Excel 97.

6.2. APO B-100 TRANSGENIC MOUSE STUDY

6.2.1. Animals

Male apo B-100 transgenic mice (C57BL/6NTac-TgN), aged 5-9 weeks, were obtained from Taconic (Germantown, NY). Animals were housed in cages of standard dimensions with 3 animals per cage. Animals were kept in an a room with constant temperature (19-23°C) and relative humidity (45-65%) with a 12/hour day/night cycle in which non-recycled filtered air was changed approximately 10 times per hour.

6.2.2. Cholesterol Diet

In order to compare the effects of FM-VP4 in the apo B-100 transgenic mouse to those in the apo E-deficient mouse previously reported,\textsuperscript{94} the same diet composition was used: 0.2% (wt/wt) cholesterol and 9% (wt/wt) fat. The diet was prepared and pelleted into ½” pellets by Harlan Teklad (Madison, WI).
6.2.3. Drug Administration

Tap water alone or tap water containing 2% (wt/vol) FM-VP4 was made available *ad libitum* in a glass feeder bottle with a stainless steel nipple. The drinking water was prepared and changed weekly. The 2% (wt/vol) FM-VP4 dose was also chosen to parallel the study design of the apo E-deficient mice in which administration of 2% (wt/vol) and (wt/wt) FM-VP4 in water and food reduced plasma cholesterol and triglycerides significantly and prevented the development of atherosclerotic lesions.94

6.2.4. Study Design

The purpose of the first part of this study was to measure the expression of the ABCA1, ABCG5 and ABCG8 genes after short-term administration of FM-VP4.

The preliminary study involved 6 groups with 3 animals in each group. Groups were as follows:

- Group 1: cholesterol diet control
- Group 2: cholesterol diet and FM-VP4 2% (wt/vol) in water
- Groups 3 & 4: regular mouse chow for 3 and 7 days respectively
- Groups 5 & 6: regular mouse chow and 2% (wt/vol) FM-VP4 in water for 3 & 7 days respectively

Allocation to each group was randomly determined before the start of the study. Homogeneity of the groups was validated on the criteria of body weight, total cholesterol and triglycerides on the day of randomization.

Upon arrival, animals were acclimatized for 7 days before treatment was initiated. After 3 and 6 days of treatment (days 4 and 7) with FM-VP4, 3 mice in the control group (fed regular mouse chow) and 3 mice in the treated group were euthanized and livers and small intestines were collected for mRNA (ABCA1, ABCG5 and ABCG8) analysis. Blood was collected by cardiac puncture.
The remaining mice were fed a diet containing 0.2% cholesterol with 9% fat (test diet) with 3 mice in the treatment group being fed 2% FM-VP4 in water and 3 mice in the control group receiving regular tap water. These mice were weighed and bled every 2 weeks. At the end of the experiment (week 18), remaining mice were euthanized, livers and small intestines were collected and flash frozen in liquid nitrogen and stored at -80°C. Hearts were harvested and perfused and stored in 10% buffered formalin and blood was collected by cardiac puncture into EDTA tubes.

6.2.5. Blood Sampling

Each mouse was restrained in a 50-ml plastic Falcon tube and blood was drawn from the tail vein (~150μl) into EDTA capillary tubes. Blood samples were then centrifuged at 3000rpm for 10 minutes at 4°C and plasma was removed and stored at -20°C until analysed.

6.2.6. Lipid Analysis

Plasma total cholesterol and triglycerides were measured using enzymatic reagents (Sigma-Aldrich). Apo B levels were measured by an immunoturbidimetric assay kit (Wako Diagnostics).

6.2.7. Atherosclerotic Lesion Analysis

Subsequent to animal sacrifice, hearts were washed out with 10% buffered formalin and then stored in fresh 10% buffered formalin. Tissues surrounding the aorta including all fat were trimmed, hearts were frozen in liquid nitrogen and the aorta was cut transversely at the aortic root in 10 μM sections. Slides were stained with oil red O, Movat's pantachrome and hematoxyllin-eosin. The lesional area was quantified by using a computer aided image-analysis system (Image-Pro Plus, Spot Diagnostic Instruments Inc.) with a magnification factor of X 6 in a blinded fashion. Each slide had three sections and the measurements were done twice; the means were subjected to statistical analysis.
6.2.8. Gene Expression Analysis

Liver and jejunum tissues were homogenized in Trizol® reagent from Invitrogen Life Technologies (Burlington, ON) to isolate total RNA. Reverse transcription polymerase chain reaction (RT-PCR) was performed to create a cDNA copy of the RNA. Presence of the characteristic 18s and 28s RNA strands was then confirmed by gel electrophoresis. Portions of the cDNA were subsequently amplified in the Roche Applied Science (Laval, QC) Lightcycler® using gene-specific primers and the SYBR Green I detection method. Amplification products of gene-specific regions were compared to amplification products from porphobilinogen deaminase (PBGD) and quantified using Roche’s RelQuant software.

6.2.9. Statistical Analysis

Continuous factors were compared using two-tailed student’s t-test with unequal variance. Data are presented as means ± standard deviations. Values were considered significant if p<0.05. All data were analysed using the basic statistical functions provided in Microsoft Excel 97.
7. **RESULTS**

7.1. **HYPERCHOLESTEROLEMIC RABBIT STUDY**

7.1.1. 0.5% Cholesterol Diet

After 7 days of cholesterol feeding on the 0.5% (wt/wt) cholesterol diet, average plasma cholesterol levels in the rabbits increased by more than 10-fold from 0.9mmol/L to 10.9mmol/L. After drug treatment was initiated, plasma TC levels continued to rise. At the end of week two, treatment groups receiving 1mg/kg & 5mg/kg FM-VP4 demonstrated a significant decrease in plasma TC compared to control (figure 3). Due to the extremely high cholesterol levels and signs of illness in some animals, however, cholesterol feeding and treatment were suspended after week 2.

![Figure 3. Plasma total cholesterol: 0.5% cholesterol diet.](image)

*Figure 3. Plasma total cholesterol: 0.5% cholesterol diet.*

*Dose-response of VP4 in rabbits fed a 0.5% cholesterol diet.*

*p<0.05

Animals that were originally excluded from the study after the first week of cholesterol feeding and subsequently washed out (fed only regular rabbit chow) for a 5-week period were then fed a diet containing 0.05% (wt/wt) cholesterol. After 2 weeks on this diet, however, there was no significant increase in plasma TC. When the dietary cholesterol
concentration was increased to 0.1% (wt/wt), an appreciable increase in plasma TC was observed (figure 4).

**Figure 4.** Plasma total cholesterol: diets with varying cholesterol concentrations.

*Fluctuations in plasma total cholesterol levels are shown as dietary cholesterol concentration is modified.*
7.1.2. 0.1% Cholesterol Diet

The rest of the animals were washed out for 8 weeks after which a new baseline was determined and FM-VP4 treatment was restarted after hypercholesterolemia was re-induced through the feeding of 0.1% (wt/wt) cholesterol diet for 1 week. In all of the treatment groups as well as in the control group, plasma cholesterol levels continued to rise over the first 3 weeks of the study. Between weeks 3 and 6, all groups, including control, demonstrated a decrease or levelling off of cholesterol levels after which a slight increase was seen between weeks 6 and 8 and then, perplexingly, cholesterol levels in the control animals dropped while they increased in those receiving the highest dose of FM-VP4 (figure 5).

Figure 5. Plasma total cholesterol: 0.1% cholesterol (CH) diet in rabbits.
In order to examine the relative change of cholesterol and LDL-C levels within the various groups, we also looked at the percent change within each group between week 0 and each subsequent week. This allows us to look at the longitudinal effect of each dose of FM-VP4 (figures 6 & 7). Note that week 0 denotes one week of cholesterol-feeding having been completed. The total cholesterol and LDL-cholesterol levels are significantly decreased at week 6 compared to week 0 for animals fed 100mg/kg FM-VP4. Because we didn't adjust p-values for multiple comparisons, however, this may be a statistical artifact.

Figure 6. Percent change in mean plasma total cholesterol: 0.1% cholesterol diet in rabbits.

In order to compare data within groups (as opposed to between groups as shown in Figures 4 & 5), percent change was calculated each week against week 0 (subsequent to 1 week of cholesterol feeding) measurements. Data are shown as relative change from week 0. *p<0.05
Figure 7. Percent change in mean plasma LDL-C: 0.1% cholesterol diet in rabbits.

*p<0.05

During the 10-week period, plasma HDL-C decreased slightly in all groups between weeks 2 and 4 but no significant change was observed throughout the duration of the study (Figure 8).
Figure 8. Plasma HDL-cholesterol: 0.1% cholesterol diet in rabbits.

Similarly, plasma triglycerides decreased in all groups subsequent to initiation of cholesterol feeding but underwent no significant changes in any of the groups throughout the rest of the study (Figure 9).

Figure 9. Plasma triglycerides: 0.1% cholesterol diet in rabbits.
7.2. APO B-100 TRANSGENIC MOUSE STUDY

7.2.1. Plasma Lipids

After 18 weeks on the 0.2% cholesterol diet and FM-VP4 treatment, there was a significant 61% decrease in plasma total cholesterol levels which was already apparent at week 2 (figure 10).

**Figure 10. Plasma total cholesterol: apo B-100 transgenic mice.**

*p<0.05

**p<0.0005
There was also a significant 34% decrease in plasma apolipoprotein B levels (figure 11) demonstrating the profound effect of FM-VP4 on lowering non-HDL cholesterol.

Figure 11. Plasma total apolipoprotein B: apo B-100 transgenic mice.

*p<0.01  
**p<0.001
There was, however, no significant change in plasma triglyceride levels (figure 12) throughout the duration of the study period.

Figure 12. Plasma triglycerides: apo B-100 transgenic mice.
7.2.2. Atherosclerotic Lesion Analysis

Representative sections of aortic arches from control and treated mice are shown in figure 13. Figure 13a demonstrates a typical sample from a control animal. Lipid deposits are stained red and we see the development of fatty lesions in several areas along the interior of the vessel wall. In figure 13b we see a marked lack of red staining in the wall of the aortic arch. While the lesion area in control animals is not overwhelming, it is, nonetheless, significantly greater compared to the lesion area in the treated group as measured by computer-assisted image analysis (figure 14).

Figure 13. Aortic arch in apo B-100 transgenic mice.

Representative cross sections of aortic arches from apo B-100 transgenic mice on a 2% cholesterol diet for 18 weeks. 12a: control. 12b: treated with 2% FM-VP4 in water.
**Figure 14. Fatty lesion area in aortic arch.**

*p = 0.0001
7.2.3. Gene Expression Analysis

The relative expression of four different lipid-regulating proteins (ABCA1, ABCG5, ABCG8 and LDL receptor) was quantified to attempt to explain the mechanism through which FM-VP4 exerts its lipid-lowering effect. Results from these analyses revealed different effects depending on the gene being examined and its source of expression.

Figure 15 shows that although there was no significant measurable effect of FM-VP4 on ABCA1 expression in either the liver or the small intestine of the cholesterol-fed apo B-100 transgenic mice, there seemed to be a trend towards lowering of ABCA1 expression in the FM-VP4-treated mice.

![Figure 15. ABCA1 expression.](image)

Relative expression of ABCA1 in the liver and small intestine of cholesterol-fed apo B-100 transgenic mice.
ABCG5 expression in the liver, however, does show a significant reduction in response to FM-VP4 treatment whereas there is no change in its expression in the small intestine (figure 16).

Figure 16. ABCG5 expression.

Relative expression of ABCG5 in the liver and small intestine of cholesterol-fed apo B-100 transgenic mice.

*p<0.05
In both the liver and small intestine, ABCG8 demonstrated no significant change in response to FM-VP4 treatment (Figure 17). In both cases, however, a trend towards lowering is observed.

![Graph showing expression of ABCG8 in liver and small intestine](image)

**Figure 17. ABCG8 expression.**

Relative expression of ABCG8 in the liver and small intestine of cholesterol-fed apo B-100 transgenic mice.

When examining the degree of expression of both ABCG5 and ABCG8, we see that the expression of both of these genes is higher in the small intestine than in the liver. This would be expected since the bioavailability of most plant sterols is much lower and therefore little actually reaches systemic circulation and needs to be handled by the liver. The small intestine however encounters all of the phytosterols that are ingested and therefore it would be expected that ABCG5 and ABCG8, which transport sterols, including plant sterols, out of the cell, are expressed to a greater degree in the small intestine.
LDL receptor expression, on the other hand, remains unchanged in the liver while it appears to show a trend towards increased expression in the small intestine (Figure 18).

**Figure 18. LDL receptor expression.**

*Relative expression of LDL receptor in the liver and small intestine of cholesterol-fed apo B-100 transgenic mice.*
8. DISCUSSION

8.1. HYPERCHOLESTEROLEMIC RABBIT STUDY

8.1.1. Induction of Hypercholesterolemia by Dietary Means

Inducing hypercholesterolemia in the rabbit model by dietary means is a well-established method. The amount of dietary cholesterol administered, however, varies, depending on the degree of hypercholesterolemia desired and the goals of the research being carried out. Several researchers have used 0.5% (wt/wt) dietary cholesterol to induce hypercholesterolemia in the rabbit. After 60 days of treatment with a 0.5% (wt/wt) cholesterol diet, animals studied by Ikeda et al. demonstrated plasma total cholesterol levels of approximately 33mmol/L. When Ntanios et al. used this same dietary cholesterol concentration, their rabbits achieved mean plasma TC of 29.6mmol/L. In studies using 0.2% (wt/wt) dietary cholesterol to induce hypercholesterolemia in rabbits, results have shown plasma TC levels ranging from 4.0mmol/L after 2 weeks to 10mmol/L after 6 weeks of treatment. In diets with as high as 1% (wt/wt) cholesterol fed to rabbits, plasma TC was up to 56mmol/L after 12 weeks. In the present study, a dietary cholesterol concentration of 0.5% (wt/wt) was initially used to induce hypercholesterolemia in rabbits. After 2 weeks of treatment, our rabbits achieved plasma total cholesterol levels up to 43mmol/L. This dramatic increase in plasma cholesterol was accompanied by signs of illness in rabbits from all treatment groups. No comment on animal health was made in any of the other reports. Once the 0.5% (wt/wt) cholesterol diet was replaced with regular chow, signs of illness disappeared in all of the animals. In order to determine a more healthy level of hypercholesterolemia in the rabbits, we re-introduced dietary cholesterol at a concentration of 0.05% (wt/wt). When no appreciable increase in plasma TC was measured after 2 weeks on this diet, we increased the dietary cholesterol concentration to 0.1% (wt/wt). At this concentration, animals exhibited a moderate degree of hypercholesterolemia with no accompanying signs of illness throughout the duration of the study.
8.1.2. Drug Administration

Several studies have been conducted in the hypercholesterolemic rabbit model to test the lipid-lowering and antiatherosclerotic effects of oral administration of phytosterols. When rabbits on a 0.5% (wt/wt) cholesterol diet were concurrently fed either 0.5% (wt/wt) β-sitosterol or β-sitostanol, there was a marked (30%), although not significant, reduction in plasma total cholesterol subsequent to a 60-day period of β-sitostanol treatment. When these same researchers decreased the dietary cholesterol concentration to 0.2% (wt/wt) while maintaining a dietary concentration of 0.5% for the phytosterols, a statistically significant reduction of plasma TC was observed in the β-sitostanol-treated animals compared to control after 42 days of treatment. In examining the effects of increasing concentrations of dietary sitostanol treatment in rabbits fed 0.5% (wt/wt) cholesterol, a significant (49%) decrease in plasma TC was only reported in the group fed 0.8% sitostanol while no significant change was seen in those groups fed 0.01% or 0.2% (wt/wt) sitostanol. When rabbits on a 0.2% (wt/wt) cholesterol diet were treated with stanol fatty acid esters, a significant 58% reduction in plasma total cholesterol was observed in animals fed 1.2% (wt/wt) stanol fatty acid esters while no significant reduction was seen in those animals fed either 0.2%, 0.33% or 0.66% (wt/wt) stanol esters. Worth noting is that, in this same study, doubling the concentration of stanol fatty acid esters to 2.4% (wt/wt) had no additional impact on plasma total cholesterol levels.

Previous studies from our research group have demonstrated that the lipid-lowering effects of FM-VP4 are greater than the effects of either of its parent compounds administered either separately or together. Because of this, we chose doses for our initial study (0.5% (wt/wt) CH diet) that were more feasible for human drug administration. When these doses (reported as mg FM-VP4/kg body weight) are converted, based on average rabbit body weight and food consumption, to percentages of food consumed for the purpose of comparison with other studies, we can see that our doses are notably smaller than those doses used in other studies (Table 4). This may explain why, despite the greater potency of FM-VP4 compared to phytostanols, we still see no appreciable effect of the drug at the concentrations administered with the 0.5% CH diet. Based on the data from these other studies, the significant reduction of total plasma cholesterol observed at week 2 (Figure 3)
in animals receiving 1mg/kg and 5mg/kg FM-VP4 are likely not due to the effect of FM-VP4 since no effect is seen at higher doses of the drug. These effects are rather more likely due to variations in plasma total cholesterol levels between animals (n=3 in each group) because of differences in responses to cholesterol feeding and drug treatment.

<table>
<thead>
<tr>
<th>Dose administered (mg FM-VP4/kg body weight)</th>
<th>Dose percent of total food consumed</th>
<th>Cholesterol:FM-VP4 administered (wt:wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.5% cholesterol diet</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1mg/kg</td>
<td>0.002%</td>
<td>250:1</td>
</tr>
<tr>
<td>5mg/kg</td>
<td>0.01%</td>
<td>50:1</td>
</tr>
<tr>
<td>20mg/kg</td>
<td>0.04%</td>
<td>12.5:1</td>
</tr>
<tr>
<td>50mg/kg</td>
<td>0.1%</td>
<td>5:1</td>
</tr>
<tr>
<td><strong>0.1% cholesterol diet</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25mg/kg</td>
<td>0.05%</td>
<td>2:1</td>
</tr>
<tr>
<td>50mg/kg</td>
<td>0.1%</td>
<td>1:1</td>
</tr>
<tr>
<td>100mg/kg</td>
<td>0.2%</td>
<td>1:2</td>
</tr>
</tbody>
</table>

**Table 4. Conversion of doses of FM-VP4 administered to rabbits.**

*Doses of FM-VP4 administered to rabbits are converted to percent of total food consumed as well as ratio to cholesterol consumed.*

Because of these observations, when we reduced the dietary cholesterol concentration to 0.1% (wt/wt) we also increased the dose of FM-VP4 administered. With the hypothesis that FM-VP4 functions by competitively inhibiting the incorporation of cholesterol into the mixed micelle, we decided to examine concentrations of FM-VP4 that would more likely be able to compete with the concentration of dietary cholesterol being consumed. Additionally, these new doses are still lower than those used in studies with phytostanols alone but are in a range that is more comparable based on the relative amounts of dietary cholesterol being concomitantly consumed.

8.1.3. Plasma Lipids

When dietary cholesterol concentration was reduced from 0.5% to 0.1% (wt/wt), there is still no significant change observed in plasma total cholesterol levels compared to the control. What is perhaps most interesting about the data obtained in this study is that, in
looking at Figure 5, we can see what appears to be a dose-response trend between the three treatment groups beginning at week 6 but it is the control group that is puzzling. We would intuitively suspect that plasma TC levels in control animals would continue to rise throughout the study as is seen in other studies but here they seem to level off at week 2 and even decrease at week 10. Again, with only 3 animals in each group, and such large standard deviations in each group, it is difficult to observe any trends from this data. In other studies in the hypercholesterolemic rabbit model, researchers have similarly reported large degrees of variation between animals which often leads to the observation of trends but not to statistically significant conclusions.83, 84

In making within-group comparisons, instead of between-group comparisons, however, a more readily observable trend is apparent. Figures 6 and 7 show the percent change within treatment groups in total cholesterol and LDL-cholesterol compared to week 0 respectively. In this case, beginning at week 4 for total cholesterol and week 3 for LDL-C, we start to see a clearer dose-response trend although the only statistically significant decrease is seen in the 100mg/kg group at week 6. This result may be a statistical artifact since we didn’t adjust our p-values for multiple comparisons therefore the likelihood of a significant result due to chance is increased. Again, it is puzzling to observe that both total cholesterol and LDL-cholesterol values seem to decrease between weeks 2 and 10 in the control group.

The great disparity within groups is also very apparent in the high standard deviation observed in all groups at different time points. Several factors may contribute to this deviation including differences in response in animals to cholesterol feeding and drug administration as well as the fact that in order to obtain a dietary cholesterol concentration of 0.1%, 0.5% (wt/wt) cholesterol pellets were mixed with regular chow. This resulted in a diet heterogeneous in both appearance and, assumedly texture since the pellets containing cholesterol were of different dietary composition and size. A qualitative observation was made that some rabbits tended to prefer one pellet type over the other although it was observed that there was not an appreciable difference to this effect between groups.
8.2. APO B-100 TRANSGENIC MOUSE STUDY

8.2.1. Plasma Lipids

As can be seen as early as week 2 in this study, FM-VP4 exerts a profound effect on lowering plasma cholesterol levels in the cholesterol-fed apo B-100 transgenic mouse model. By the 18th week of the study, we demonstrated a 61% decrease in plasma total cholesterol compared to controls (13.8mmol/L in control mice to 5.3mmol/L in treated mice) (figure 10). The values that we measured are very similar to those measured in other laboratories where a total cholesterol level of 8mmol/L was shown at week 5 when animals were on a 1.25% (wt/wt) cholesterol diet whereas we demonstrated a mean total cholesterol of 8.4mmol/L at week six in animals fed 0.2% (wt/wt) dietary cholesterol.106 At week 16, we demonstrated a 34% decrease in plasma apolipoprotein B levels from 1.58g/L in the control mice to 1.04g/L in treated mice (figure 11). Our results are also similar to those measured by Purcell-Huynh et al. where they showed apo B levels at 1.20g/L at week 5 and we had a mean value of 1.12g/L at week 4.106 These results indicate that FM-VP4 significantly lowers both serum total and non-HDL cholesterol levels in this model.

8.2.2. Atherosclerotic Lesion Analysis

In previous studies conducted in the apo B-100 transgenic mouse, it has been shown that the mice develop extensive lipid-rich atherosclerotic lesions throughout the proximal 1200μm of the aorta after 18 weeks when fed a diet containing 1.25% (wt/wt) cholesterol and 16% (wt/wt) fat.106 In our study, we used a diet containing only 0.2% (wt/wt) cholesterol. This may be the reason for which we did not see the extent of fatty lesions in the aortic arch that have been previously reported. Nonetheless, we demonstrated a statistically significant reduction in fatty lesion area in the aortic arch of the FM-VP4-treated animals compared to controls (figure 14).
8.2.3. Gene Expression Analysis

8.2.3.1. ABCA1

In order to attempt to explain the molecular mechanism through which FM-VP4 lowers plasma cholesterol levels, we measured the relative expression of particular genes which are involved in cholesterol transport across membranes. Recent work by Plat and Mensink (2002) has shown that, in caco-2 cells, ABCA1 expression was induced when cells were treated with mixed micelles containing sitostanol or cholesterol plus sitostanol thereby suggesting that plant stanols increase enterocyte ABCA1-mediated cholesterol efflux back into the intestinal lumen.\textsuperscript{110} We therefore hypothesized that the expression of ABCA1 may be increased in response to FM-VP4 treatment in the apo B-100 transgenic mouse model thereby resulting in decreased cholesterol absorption. In examining the pattern of relative expression of ABCA1 (Figure 15), however, we can see that while there is no statistically significant difference between expression in control and treated animals, there is an apparent trend towards decreased expression in the treated mice in both the liver and small intestine. This may mean that instead of inhibiting cholesterol absorption by increasing ABCA1's activity in transporting cholesterol out of the cell, the decreased amount of cholesterol made available to the cell leads to a decrease in the necessity for the function of ABCA1 and therefore a decrease in its cellular expression. Similar results were demonstrated with an analogue of the cholesterol absorption inhibitor ezetimibe.\textsuperscript{121}

8.2.3.2. ABCG5 and ABCG8

ABCG5 and ABCG8 are recently-described members of the ABC transporter superfamily that have been shown to be involved in regulating cellular sterol content through the transport of cholesterol and phytosterols.\textsuperscript{107} Previous studies have shown that increased entry of cholesterol into the enterocyte leads to increased expression of ABCA1, ABCG5 and ABCG8.\textsuperscript{111} ABCG5 and ABCG8 have been shown to be expressed in both the liver and the intestine.\textsuperscript{107} Our studies demonstrated that ABCG5 expression is significantly decreased in the liver while the hepatic response of ABCG8 is a trend towards lowering which approaches but does not achieve statistical significance (figures 16 & 17). Previous studies from our
research group have demonstrated a very low bioavailability for FM-VP4. For this reason, any effect that FM-VP4 exerts on lipid-lowering is thought to occur presystematically. The effect of FM-VP4 that we see on hepatic ABCG5 and ABCG8 expression is most likely secondary to its lipid-lowering capabilities: with decreased plasma cholesterol there is a decrease in the concentration in the hepatocyte and therefore a decreased need to transport cholesterol out of this tissue. Because of the very low bioavailability of FM-VP4 and phytostanols in general, it is unlikely that there is any direct effect of this drug on hepatic ABCG5 and G8 expression. In examining the expression pattern of these transporters in the small intestine, we can see that there is no change in ABCG5 expression in the small intestine while there appears to be an insignificant trend towards lowering of ABCG8 in this tissue. If our hypothesis for ABCA1 holds true and a decreased concentration of cholesterol actually enters the enterocyte, we would again expect that ABCG5 and ABCG8 would be downregulated in response to FM-VP4 treatment whereas the increased concentration of cholesterol provided in the control diet would increase the expression of these genes. This appears to be the case with ABCG8 in the small intestine. A noteworthy finding with respect to intestinal ABCG5 expression is that its degree of expression is much higher than the expression of all of the other genes analyzed. While we might expect this in the treated animals due to an increased concentration of phytostanols in the small intestine provided by FM-VP4, it would then follow that the expression of ABCG5 in the control animals would not be accordingly high. This, however, is not the case and further studies are needed to fully elucidate this phenomenon.

8.2.3.3. LDL Receptor

The LDL receptor is another key protein involved in cholesterol homeostasis. When the cellular demand for cholesterol is high, the expression of this protein is increased to accommodate this demand. In examining the relative expression patterns of the LDL receptor in the liver and small intestine of our cholesterol-fed, FM-VP4-treated apo B-100 transgenic mice, we see that in the liver the expression pattern in treated animals remains unchanged compared to controls while there is an appreciable, although not significant increase in LDL receptor expression in the small intestine. This expression pattern is interesting for two reasons. First of all, we know that in response to decreased plasma
cholesterol concentrations, the liver compensates by synthesising endogenous cholesterol. For this reason, the hepatic cholesterol pool is maintained, even when it is not taking up exogenously-derived cholesterol and therefore has no need for changing the rate of uptake of LDL. This would explain why hepatic LDL receptor expression remains unchanged in response to FM-VP4 treatment (figure 18). The second reason for which the LDL receptor expression pattern in this study is interesting is that in the small intestine, there is a decrease in the amount of cholesterol being absorbed into the enterocyte subsequent to FM-VP4 treatment. In order to compensate for the decreased concentration of exogenously-derived cholesterol in the enterocyte, LDL receptor expression is upregulated in order to increase the uptake of cholesterol from the bloodstream into the enterocyte (figure 19). This then would lend strength to our hypothesis that FM-VP4 acts on the micellar incorporation of cholesterol, before cholesterol is actually absorbed by the enterocyte. While this hypothesis would provide a viable explanation for the gene expression pattern that we have observed, further studies are warranted to verify these findings.
Figure 19. Summary of proposed effects of FM-VP4.

18a: Normal  18b: FM-VP4-treated. FM-VP4 acts by inhibiting the incorporation of cholesterol into the mixed micelle thereby decreasing the uptake of exogenous cholesterol by the enterocyte. This appears to result in a concomitant decrease in the expression of the ABCA1, ABCG5 and ABCG8 genes while increasing the expression of the LDL-receptor.
9. FUTURE DIRECTIONS

FM-VP4 lowers plasma cholesterol in several animal models. The hypercholesterolemic rabbit model and the apo B-100 transgenic mouse models are both valuable models for studying hypolipidemic drugs because of their lipoprotein profile which resembles that of humans. We have demonstrated that varying dietary cholesterol concentrations in the rabbit has a dramatic effect on plasma cholesterol levels. Additionally, we have demonstrated that FM-VP4 appears to have a cholesterol-lowering effect in the hypercholesterolemic rabbit. Future studies using an increased number of animals in each group to lend greater statistical power to the data analysis as well as to account for the large degree of inter-animal variation are warranted. In future studies using this model, it would also be prudent to use a homogeneous cholesterol diet and measure the amount of food that each animal is consuming each day in order to perform subgroup analyses comparing food consumption to diet and drug response. Additionally, future studies in this model should include atherosclerotic lesion analysis and gene expression analysis to further understand the effects of FM-VP4 in the atherosclerotic process. Ultimately, future studies would include FM-VP4 and HMG-CoA reductase inhibitor co-therapy in order to target both the exogenous and endogenous cholesterol synthesis pathways simultaneously and measure whether the effect of these two drugs is an additive or a synergistic effect.

In the cholesterol-fed apo B-100 transgenic mouse model we have demonstrated a significant reduction in plasma total cholesterol and apolipoprotein B levels subsequent to treatment with 2% (wt/vol) FM-VP4 in water. These decreases were also accompanied by a small but significant decrease in atherosclerotic lesion area. Gene expression analyses in this model seem to indicate that the ABC transporters (ABCA1, ABCG5 and ABCG8) may be downregulated in response to FM-VP4 treatment as a secondary response to the decreased concentration of cholesterol entering the enterocyte and systemic circulation. Analysis of the expression of the LDL receptor indicates that in response to treatment with FM-VP4, the cell may compensate for the decreased plasma cholesterol concentration by inducing endogenous cholesterol synthesis thereby maintaining cholesterol homeostasis and not changing the expression of the LDL receptor. In the small intestine, on the other hand, the decreased cholesterol absorption seems to have led to an increase in LDL receptor
expression in order to restore the intracellular cholesterol pool. Future studies in this model are needed in order to verify these results. Future studies should undoubtedly include a greater number of animals in each group as well as a negative control group in which mice are fed a regular chow diet only in order to determine the baseline expression levels of the various lipid-regulating proteins to be analysed. It would also be helpful to include, in future studies, groups of animals with varying concentrations of both dietary cholesterol content and FM-VP4 to assess the relationship between FM-VP4 efficacy and dietary cholesterol load.

The cholesterol-lowering and atheroprotective effects of FM-VP4 shown here further substantiate the potential of its use as a novel drug for the prevention and treatment of coronary heart disease. Because it appears to act primarily at the level of the exogenous cholesterol pathway and does not appear to have systemic effects, the real value of FM-VP4 lies in its potential to be used as adjunct therapy with other lipid-lowering agents, particularly HMG-CoA reductase inhibitors which target the endogenous cholesterol pathway. Such a pharmacological combination could be very powerful and provide the additional cholesterol-lowering needed by many individuals who are not currently able to safely and effectively meet their cholesterol-lowering targets in order to reduce the risk of coronary heart disease.
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