The results of numerous clinical trials with statins and other drugs have demonstrated the principal possibility of the prevention and regression of atherosclerosis by pharmacotherapy. Using cellular models and natural products, we have developed an approach to prevent atherosclerotic manifestations in arterial cells. Based on our knowledge of atherosclerosis, we developed anti-atherosclerotic drugs. Two-year treatment with Allicor (garlic powder) has a direct anti-atherosclerotic effect on carotid atherosclerosis in asymptomatic men. Inflaminat (calendula, elder, and violet), which possesses anti-cytokine activity, has been shown to cause the regression of carotid atherosclerosis. The phytoestrogen-rich drug karinat (garlic powder, extract of grape seeds, green tea leaves, hop cones, β-carotene, α-tocopherol, and ascorbic acid) prevents the development of carotid atherosclerosis in postmenopausal women. Thus, our basic findings were successfully translated into clinical practice. Because of this translation, a novel approach to anti-atherosclerotic therapy was developed. Our clinical trial confirmed the efficacy of both the novel approach and the novel drugs.

Alexander Orekhov

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Content

Introduction

Atherosclerosis of major human arteries causes cardiovascular diseases, including myocardial infarction and stroke. Atherosclerosis develops in the arterial wall and remains asymptomatic until ischemia of distal organs is evident. Thus, first events are often fatal. Treatment of the clinical manifestations of atherosclerosis is largely aimed at reducing symptoms or affecting the hemodynamic response, and it often does not affect the cause or course of the disease, namely, the atherosclerotic lesion itself.

In epidemiological studies, hypercholesterolemia (a high level of plasma cholesterol) and a high concentration of plasma low density lipoprotein (LDL) have been significantly associated with the development of premature atherosclerosis [1]. Cholesterol accumulation in the arterial wall is the primary sign of atherosclerosis. It is generally accepted that LDL is the major source of cholesterol deposits in the vessel wall. Furthermore, the anti-atherosclerotic effects of statins (lipid-lowering drugs), that have been revealed in many clinical trials should also be considered. However, statins were never indicated simply for the direct treatment or prevention of atherosclerosis. They are used predominantly in the course of hypolipidemic therapy, and the effects of treatment with statins are estimated by success in reaching the target level of LDL cholesterol and not by the regression of the atherosclerotic lesion or intima-media thickness. The latter should be considered a beneficial effect, which is mainly due to pleiotropic mechanisms of action. In the case of direct anti-atherosclerotic therapy, treatment strategies should be concentrated on the prevention of atherosclerotic lesion growth, reduction in lipid core mass, and stabilization of the atherosclerotic plaque. Taken together, these approaches could theoretically result in the regression of lesions.

Chapter 1.
Clinical Studies on Atherosclerosis Regression

Atherosclerosis can remain asymptomatic for a long time. New insights into the disease process are difficult to obtain, and the rapid evaluation of direct anti-atherosclerotic therapies is impossible unless techniques to assess early atherosclerotic changes are used. Imaging trials constitute the only approach in the development of anti-atherosclerotic therapy to test drug efficacy.

1.1. Atherosclerosis Imaging

There are multiple imaging methods used to assess atherosclerosis [2]. Traditionally, quantitative coronary angiography has been the predominant imaging modality used to assess the progression or regression of atherosclerosis. With quantitative coronary angiography, the cross-sectional coronary anatomy is depicted as a planar silhouette of a contrast-filled vessel lumen. Both modalities are highly relevant in a clinical setting, but do not provide useful information on the early stages of arterial wall thickening before lesion formation. Intravascular ultrasound (IVUS) is an invasive imaging method that reveals both the arterial wall and lumen for the identification of the true regression of atherosclerosis. Carotid intima-media thickness (CIMT) can be measured non-invasively. The method has been well validated in histological and epidemiological studies [3]. Magnetic resonance imaging (MRI) of carotid arteries also allows for the noninvasive visualization of the arterial wall and lumen without exposing the patient to radiation, albeit with a lower image resolution compared to IVUS. MRI can also provide information about the composition of plaques. Positron emission tomography (PET) assessment of 18F-fluorodeoxyglucose uptake in the vascular wall, using computed tomography (CT) as a roadmap, can reveal the metabolic activity of cells and, potentially, inflammation in the arterial wall [4].

1.2. Carotid Intima-Media Thickness (CIMT)

Atherosclerosis affects most vascular beads, and the noninvasive imaging of superficial arteries by ultrasound has been recognized as a surrogate measure of atherosclerosis in numerous studies. Extracoronary atherosclerotic lesions can be quickly and safely evaluated in the carotids, femoral arteries, and the abdominal aorta. The grade of atherosclerosis in extracoronary sites correlates with a greater number of standard risk factors and, more importantly, with greater cardiac risk [5]. Of the peripheral arterial surrogates, carotid atherosclerosis has been the most closely correlated with coronary artery disease [6-9]. Peripheral arterial ultrasonography is regarded as a sensitive tool for the detection of early atherosclerosis and may be useful in assessing the response to therapy. The thickening of the intima-media of the arterial wall is the earliest detectable anatomic change in the development and progression of atherosclerosis. High-resolution B-mode ultrasonography is widely used for the noninvasive quantification of carotid IMT as a measure of subclinical atherosclerosis [10]. CIMT is believed to be a marker of generalized atherosclerosis and is predictive of clinical cardiovascular events [7, 9, 11-16]. Thus, ultrasound imaging of the intima-media thickening in carotid arteries is an applicable method for the monitoring of atherosclerosis during long-term treatment.

To compare the effects of different anti-atherosclerotic therapies, we selected those studies that measured the CIMT using similar design and ultrasonographic protocols. The results of these studies are described below.

1.4. Drugs Affecting Lipid Metabolism

Most studies of atherosclerosis imaging were conducted with drugs known to affect lipid metabolism. This is explained by the fact that the only hypothesis that has received confirmation in the clinic is the cholesterol hypothesis. This hypothesis was

proposed more than 100 years ago by N.N. Anitschkow. Originally, Anitschkow's hypothesis linked atherosclerosis with high total cholesterol levels in the blood. Modern theories only cover some aspects of this hypothesis; in particular, how atherosclerosis is related not to the total level of cholesterol but to the atherogenic cholesterol of low density lipoprotein and the anti-atherogenic cholesterol of high density lipoprotein is examined. Additionally, the role of various molecules involved in lipoprotein metabolism is discussed.

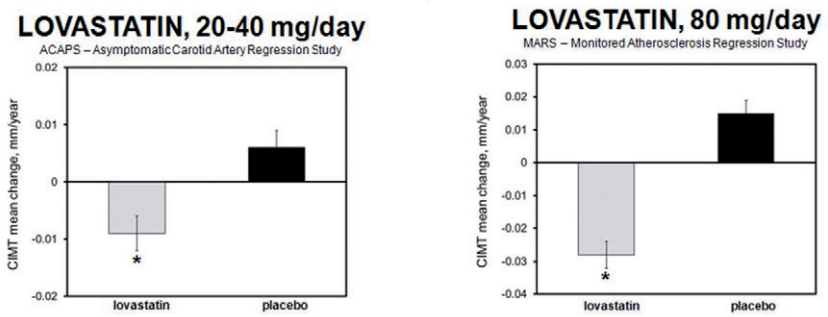

Figure **1** Anti-atherosclerotic effects of lovastatin.

1.4.1. Statins

Statins were introduced to clinical practice as drugs that reduce cholesterol synthesis and LDL level. It must be emphasized that statins were prescribed as lipid-lowering drugs, not as anti-atherosclerotic drugs. In the first atherosclerosis imaging clinical trials, statins were used as lipid-lowering agents to test the hypothesis that the lowering of cholesterol can cause the regression of atherosclerosis. Although a direct correlation between the reduction of cholesterol and the regression of atherosclerosis has

not been revealed, these studies demonstrated the anti-atherosclerotic effects of statins. Later, to explain the non-lipid, anti-atherosclerotic effects, the pleiotropic action of statins was discussed [17-24].

The first statin that demonstrated an anti-atherosclerotic effect was lovastatin [25]. Figure 1 shows the anti-atherosclerotic effects of lovastatin obtained in two studies, ACAP and MARS, with different doses of lovastatin [25, 26]. The higher dose caused a greater effect (Figure 1).

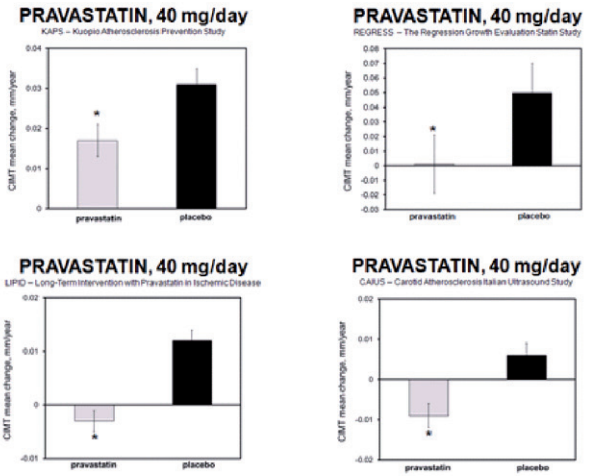

Figure **2** Anti-atherosclerotic effects of pravastatin.

Another statin, pravastatin, has been extensively studied in clinical trials with atherosclerosis imaging. The anti-hypercholesterolemic effects of pravastatin are similar to those of lovastatin [27]. However, the anti-atherosclerotic effects were absent at dosages of 20-40 mg/day, as in the study PLAC II [28], or were moderate, as in the

studies KAPS, REGRESS, LIPID, CAIUS [29-32]. In Figure 2, it is clear that in the two studies (KAPS, REGRESS), pravastatin only slowed the development of atherosclerosis, but it did not cause regression, and in the other two studies (LIPID, CAIUS), pravastatin produced a moderate regression of atherosclerosis.

The anti-atherosclerotic action of one of the most recently developed statins, rosuvastatin, has been revealed [33]. It was shown that rosuvastatin prevents the progression of atherosclerosis (Figure 3).

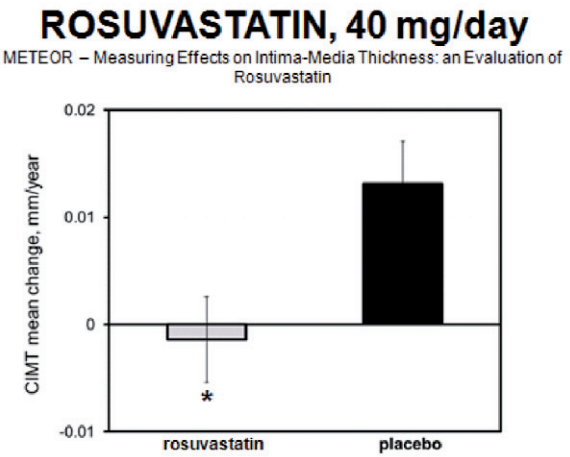

Figure **3** Anti-atherosclerotic effects of rosuvastatin.

Comparative studies of the anti-atherosclerotic efficacy of different statins were also conducted [34, 35]. The effects of atorvastatin were compared with the effects of simvastatin and pravastatin. Figure 4 shows that atorvastatin was more effective.

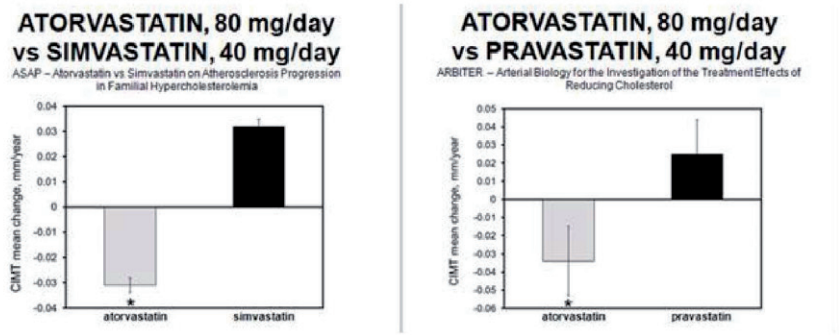

Figure **4** Anti-atherosclerotic effects of statins.

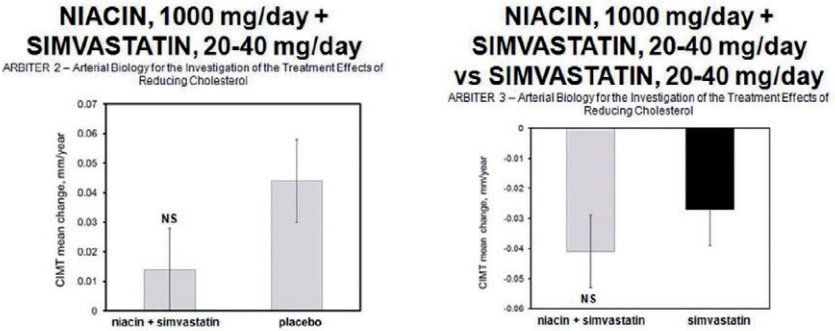

Figure **5** The anti-atherosclerotic effects of simvastatin in combination with niacin.

Statins were also used in combination with an HDL-raising agent, nicotinic acid [36-38]. The combination of simvastatin with niacin did not induce atherosclerosis regression and did not have statistically significant anti-atherosclerotic effects compared to placebo (Fig. 5). In the regression of atherosclerosis, this combination was not significantly more effective than simvastatin alone (Fig. 5). Another comparative-

effectiveness trial showed that the use of extended-release niacin caused a significant regression of carotid intima-media thickness when combined with a statin (simvastatin or atorvastatin) and that niacin is superior to ezetimibe (Fig. 6).

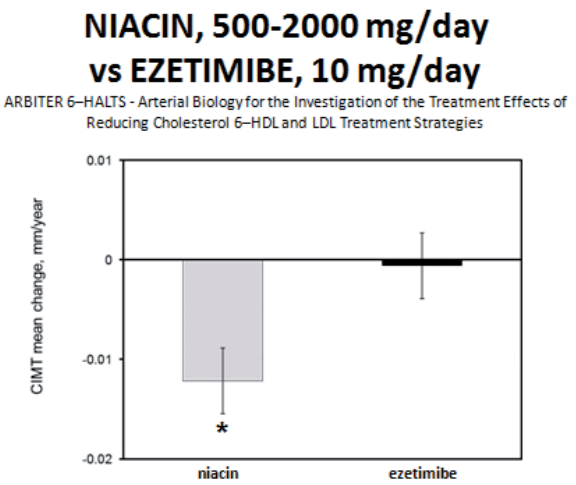

Figure **6** The anti-atherosclerotic effect of niacin.

1.3.2 HDL-Therapy

It is now widely acknowledged that designing pharmaceuticals to increase high density lipoprotein (HDL) levels is likely to be beneficial for the treatment of atherosclerosis. This hypothesis relates back to the initial finding from the Framingham Heart Study in which plasma HDL independently and inversely correlated with cardiovascular disease, irrespective of LDL levels [39]. Increasing levels of circulating HDL constitute an attractive therapeutic target.

HDL-raising therapy (HDL-therapy) may involve infusions of HDL, HDL-like

particles, or apoA-1 mimetic peptide, as well as treatment with cholesteryl ester transfer protein (CETP) inhibitors. However, clinical trials with apoA-1 Milano/phospholipid complexes (ETC-216) [40], a complex of native apoA-1 with phosphatidylcholine (CSL-111) [41] and delipidated HDL [42], did not reveal statistically significant anti-atherosclerotic effects when compared to placebo.

Another approach to raising HDL cholesterol levels is CETP inhibition. CETP transfers cholesteryl esters from HDL to the apoB-containing lipoproteins LDL and VLDL. The theoretical rationale for inhibiting CETP is based on the fact that HDL cholesterol in HDL fractions cannot cause atherosclerosis. LDL, on the other hand, delivers cholesterol to all tissues, including the vessel wall, and excess cholesterol in LDL fractions is a recognized causative factor of atherosclerosis. So far, all attempts to demonstrate the anti-atherosclerotic effectiveness of CETP inhibition were unsuccessful because all known clinical trials related to the inhibition of CETP were stopped prematurely for various reasons.

1.4. Non-Lipid Anti-Atherosclerotic Therapy

1.4.1. Calcium antagonists

The incentives for pursuing atherosclerosis imaging studies with calcium antagonists included the data on the anti-atherosclerotic effectiveness of calcium antagonists in animal models [43, 44]. However, the first-in-man trials with calcium antagonists were unsuccessful [45, 46]. In hypertensive patients, neither isradipine nor verapamil induced the regression of atherosclerosis, nor did either of them significantly differ from the diuretics used as control drugs (Fig. 7).

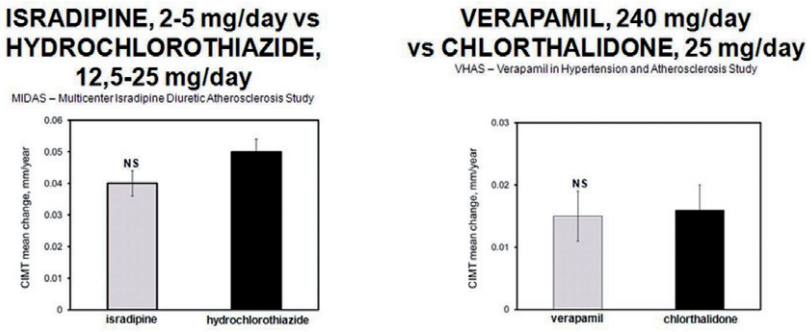

Figure **7** Anti-atherosclerotic effects of calcium antagonists.

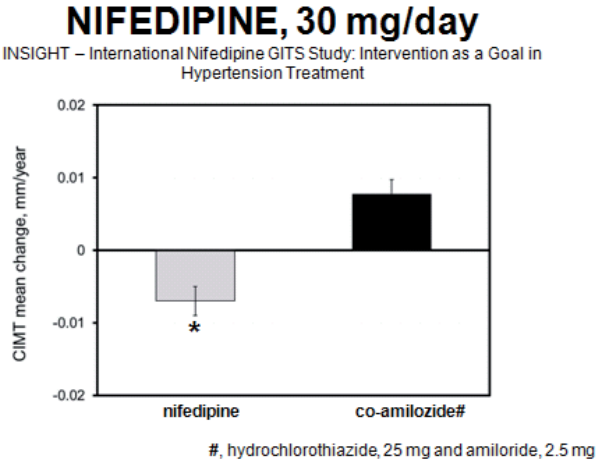

Figure **8** Anti-atherosclerotic effects of nifedipine.

The only successful trial with nifedipine [47] demonstrated the regression of atherosclerosis in hypertensive patients and a significant difference between treatments with nifedipine and diuretics, which were used as control drugs (Fig. 8).

1.4.2. Angiotensin-converting enzyme inhibition

Experimental and epidemiological data suggest that the activation of the renin-angiotensin-aldosterone system plays an important role in atherogenesis and that prolonged angiotensin-converting enzyme (ACE) inhibition may be beneficial [48]. Indeed, long-term treatment with the ACE inhibitor ramipril had a beneficial effect on atherosclerosis progression [49]. Ramipril significantly slowed the progression of atherosclerosis compared to placebo (Fig. 9).

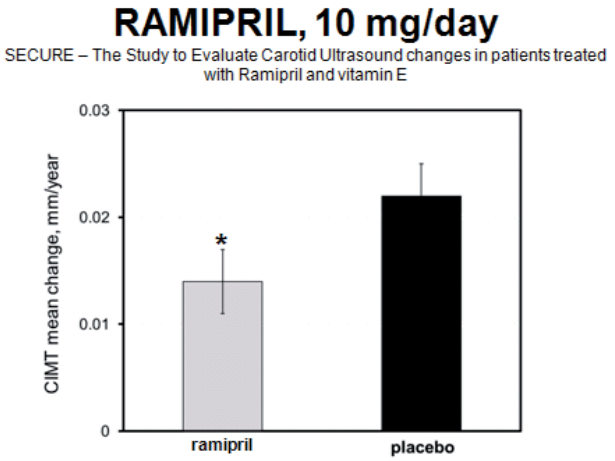

Figure **9** Anti-atherosclerotic effects of ramipril.

Chapter 2.
Development of Natural Anti-Atherosclerotic Drugs Preventing Cellular Cholesterol Retention

We believe that natural products have the potential to become the basis for creating anti-atherosclerotic drugs. On the one hand, atherosclerosis develops over many years, so anti-atherosclerotic therapy should be long-term or even lifelong. On the other hand, tachyphylaxis, long-term toxicity, and cost, amongst other issues, may present problems for the use of conventional medications over the long term. Thus, drugs based on conventional natural products can be a good alternative.

The success of clinical studies on the regression of atherosclerosis caused by statins and other drugs provoked us to develop anti-atherosclerotic drugs on the basis of natural products and to assess the effectiveness of natural drugs in atherosclerosis imaging clinical trials. The development of natural anti-atherosclerotic drugs was based on the results of our basic research of mechanisms of cellular lipidosis (cellular cholesterol retention) in human atherosclerosis.

2.1. Cellular Cholesterol Retention and Mechanisms of Atherosclerosis

Cellular lipidosis, the accumulation of cholesterol and other lipids in arterial cells, is the most prominent manifestation of atherosclerosis at the arterial cell level. In addition to lipid accumulation, the elevated proliferative activity of vascular cells and the enhanced synthesis of the extracellular matrix are characteristics of cellular atherogenesis. Collagen and glycoproteins are the main components of the extracellular matrix that form a fibrous plaque.

Intracellular lipid accumulation can be induced by LDL; however, native lipoprotein usually does not increase the cholesterol content of the cell [50]. On the

other hand, the incubation of cell cultures with chemically modified LDL results in a massive accumulation of cholesterol in the cells [50]. In vitro studies have revealed a number of atherogenic modifications of LDL, i.e., modifications that lead to cellular lipidosis [50]. These findings suggest that modified, but not native, LDL is the source of lipids that accumulate in arterial cells. Arterial intimal cells that populate atherosclerotic lesions are overloaded with lipids, and their cytoplasms are almost completely filled with lipid inclusions [51]. These cells are referred to as foam cells.

Modified LDL circulates in the bloodstream. We have discovered modified (desialylated) LDL in the blood plasma of patients with coronary atherosclerosis [52-55]. This LDL induces the accumulation of cholesterol in arterial cells [52-55]. Naturally occurring modified LDL has lower sialic acid, triglyceride and cholesterol contents; smaller particle size; greater density and negative charge; higher aggregative activity; and some other specific features [56]. We have discovered an enzyme, trans-sialydase, that is responsible for the desialylation of LDL particles in the blood [57].

In addition to desialylated LDL, more electronegative LDL and small dense LDL have been detected in human blood [58, 59]. We have provided comparative studies of LDLs that are modified in vivo. A cooperative study with an Italian group showed that the more electronegative LDL isolated by ion-exchange chromatography is desialylated LDL [60]. Desialylated LDL isolated from patient blood [52-55] is more electronegative. These findings suggest that both desialylated LDL and electronegative LDL are similar, if not identical.

We have found that a particle of desialylated LDL is smaller and denser than that of native LDL; that is, this LDL is a small dense lipoprotein [61]. On the other hand, La Belle and Krauss showed that small dense LDL has a low content of sialic acid, i.e., it is desialylated [62]. These findings point to a similarity between the two types of modified LDL.

Glycosylation is another type of in vivo LDL modification. Glycosylated LDL was found in the blood of patients with diabetes mellitus [63]. This LDL is also

atherogenic, as indicated by the fact that it induces intracellular lipid accumulation [64]. Oxidation is likely a type of atherogenic modification of LDL in vivo. However, there is no direct evidence of the presence of oxidized LDL in blood [65].

Autoantibodies are produced in response to the appearance of modified LDL in the bloodstream [66-68]. Autoantibodies to desialylated LDL react with both modified and native lipoproteins, the latter with a lesser affinity [66, 67]. The interaction between anti-LDL autoantibodies and the lipoprotein results in the formation of LDL-containing circulating immune complexes [69]. Desialylated LDL, which enters the cells as a component of immune complexes, possesses a higher atherogenic potential compared with free lipoprotein; that is, it induces a more intense cholesterol accumulation in the cell [69, 70]. The interaction with anti-LDL converts native non-atherogenic LDL into an atherogenic form, as indicated by its ability to induce intracellular cholesterol accumulation, which is accompanied by enhanced cell proliferation and extracellular matrix production [69]. We have found circulating immune complexes consisting of LDL and anti-LDL autoantibodies in the blood of most atherosclerotic patients [71-73]. A positive correlation between the levels of LDL-containing immune complexes and the severity of atherosclerosis has been demonstrated [71-75].

We have demonstrated that LDL is able to form complexes with cellular debris, collagen, elastin, and proteoglycans of the human aortic intima [76-81]. The addition of these complexes to cultured cells was shown to stimulate the intracellular accumulation of lipids. Experiments with iodinated LDL showed an increased uptake and decreased intracellular degradation of lipoproteins in complexes [80].

We showed that LDL that was modified in vivo or in vitro spontaneously self-associates under cell culture conditions, while native LDL does not self-associate [79]. A positive correlation between the atherogenic activity of modified LDL and the degree of LDL self-association has been established [79, 80]. Lipoprotein associates isolated by gel filtration were shown to induce a dramatic increase in lipid accumulation by cultured human aortic intimal cells. The removal of LDL aggregates from the incubation medium

by filtration through filters with a pore diameter of 0.1 μm completely prevented intracellular lipid accumulation [80]. Thus, self-association increases the atherogenic potential of LDL.

We can conclude that the formation of large complexes (self-associates, immune complexes, and complexes with connective tissue matrix) by modified LDL leads to intracellular lipid accumulation through enhanced cellular uptake and slow intracellular degradation of lipoprotein particles.

2.2. Anti-Atherogenic and Anti-Atherosclerotic Drugs

Taken together, our data allowed us to identify possible targets for anti-atherosclerotic therapy. The first target (target 1) is atherogenic modification (desialylation) of the LDL particle in blood. The prevention of LDL modification may be an approach to anti-atherosclerosis therapy. The second approach may be the selective removal of modified LDL from blood (target 2). The third approach may be based on the prevention of modified LDL accumulation in arterial cells (target 3). Additionally, another approach is the removal of excess lipids from foam cells (target 4). Figure 10 schematically represents these four targets. We have used all four approaches to anti-atherosclerotic therapy and believe that the most suitable approach is the third, namely, the prevention of modified LDL accumulation in arterial cells. Below, we describe the application of this approach for the development of anti-atherosclerotic therapy.

Agents capable of preventing atherogenesis are anti-atherogenic drugs, and agents that promote the regression of atherosclerotic manifestations are anti-atherosclerotic drugs. The prevention of intracellular lipid accumulation accompanied by the stimulation of arterial cell proliferation and massive extracellular matrix production may be regarded as anti-atherogenic (preventive). In terms of arterial cells, any drug effect that does not directly prevent the conversion of a normal cell into an atherosclerotic cell

(foam cell) is regarded as an indirect anti-atherogenic action. Only a drug that exhibits its preventive activity at the arterial level is a direct anti-atherogenic drug. At the arterial cell level, a drug with a direct anti-atherosclerotic action should induce the regression of the major cellular manifestations of atherosclerosis, such as reduce the intracellular lipid content, suppress cell proliferation and inhibit extracellular matrix production.

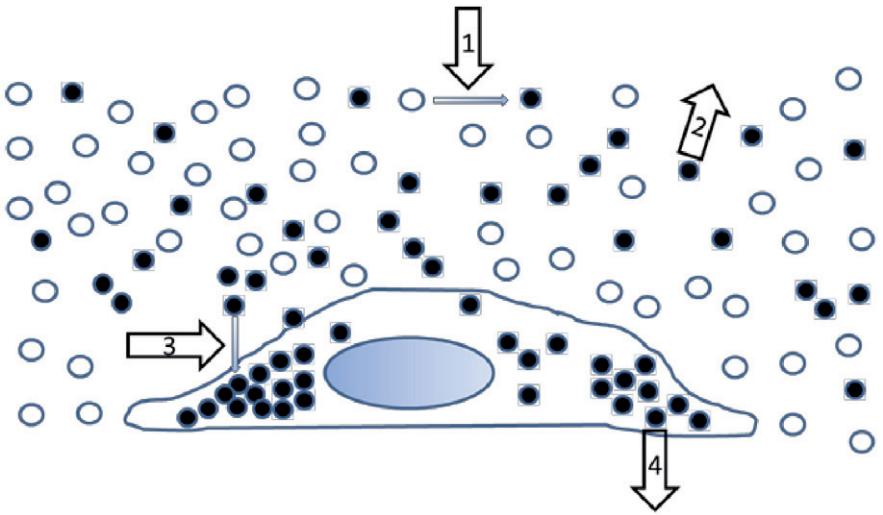

Figure 10. Targets of anti-atherosclerotic and anti-atherogenic drug actions.
Solid circles, multiple modified LDL; open circles, native LDL. Arrows indicate possible targets for anti-atherosclerotic therapy.

Thus, the drugs that affect atherosclerosis can be divided into 3 groups: (1) anti-atherosclerotic, (2) direct anti-atherogenic, and (3) indirect anti-atherogenic.

2.3. Cellular Model

In our model, we use primary culture of human aortic cells for the screening of potential drugs, the investigation of their mechanisms of action, and the optimization of anti-atherosclerotic drug therapy.

Cells are isolated from the subendothelial part of the human aortic intima, the part of aorta that is localized between the endothelial lining and the media [82]. The intima of the adult human aorta is a well-marked formation. The thickness of a normal intima varies from 50 to 120 μm [82]. Such a thickened intima is called a diffuse intimal thickening [82]. This term underlines its essential difference from very thin intimas of animal and adolescent arteries. An unaffected intima of the adult human aorta contains 10-12 lines of subendothelial cells [82].

Using collagenase and elastase, cells are isolated from the subendothelial layer of the intima of both normal and atherosclerotic parts of the aorta [83-85]. Employing formal criteria, the cells cultured from the intima can be classified as cells of smooth muscle origin. These cells are stained with antibodies to smooth muscle myosin [83-85]. For further identification of cultured cells antibodies to smooth muscle alfa-actin we used [86]. According to our calculations, primary culture of subendothelial aortic cells contains approximately 90% of smooth muscle cells that interact with antibodies to smooth muscle alpha-actin. In addition, cells cultured from the subendothelial part of uninvolved intima have ultrastructural features that are characteristic of smooth muscle cells, namely, the basal membrane and filament bundles with dense bodies [83-85]. The culture on which our experiments are performed is represented by a mixed population of typical and modified smooth muscle cells previously characterized in the human aorta [82].

Cells of the subendothelial intima isolated from atherosclerotic lesions retain all major characteristics of atherosclerotic cells when cultured. Cell cultures from fatty streaks and fatty infiltration zones have an enhanced proliferative activity [87], higher

than that of cells cultured from unaffected intima [87, 88].

Table **1** Substances tested on cellular model.

Agent	References
ANTI-ATHEROSCLEROTIC	
Cyclic AMP elevators	88, 90-93
Prostacyclin	88, 94-98
Prostaglandin E_2	88, 94, 99
Artificial HDL	100
Antioxidants	88
Calcium antagonists	88, 97, 101-104
Trapidil and trapidil derivatives	105, 106
Lipoxygenase inhibitors	99
Lipostabil	88
Mushroom extracts	107
PRO-ATHEROGENIC	
Beta-blockers	103, 108
Thromboxane A_2	97, 98
Phenothiazines	88
INDIFFERENT	
Nitrates	103
Cholestyramine	88

Many cells cultured from atherosclerotic lesions are so-called foam cells, which contain numerous inclusions, likely lipid droplets, that fill the entirety of the cytoplasm [84]. The bulk of excess lipids in foam cells consists of free cholesterol and cholesteryl

esters [84]. It should be noted that the content and composition of lipids in cultured cells within the first 10-12 days in culture remain unchanged and correspond to the respective indices of freshly isolated cells [84, 88].

Cells cultured from the subendothelial intima are capable of synthesizing collagen, proteoglycans and other components of the extracellular matrix [89].

Thus, cells isolated from an atherosclerotic lesion of the human aorta retain all the main properties that are characteristic of atherosclerotic cells when grown in culture. They exhibit an enhanced proliferative activity, contain excess cholesterol in the form of intracellular inclusions, and synthesize the extracellular matrix. These characteristics allow us to regard a culture of atherosclerotic cells as a convenient model for the investigation of the effects of various agents on atherosclerotic manifestations [88]. The investigations are carried out directly on exactly those cells that require a therapeutic action in vivo.

Using this model, we have examined the effects of different drugs and chemicals. By now, many substances have been tested [88]. The effects of several substances are summarized in Table 1. Some of the substances elicited anti-atherosclerotic effects in culture, others proved ineffective in this respect, and still others stimulated the development of atherogenic processes.

2.4. Effects of Cardiovascular Drugs in Cellular Model

Three classes of cardiovascular drugs, calcium antagonists, beta-blockers and nitrates, have been tested using our cellular model. These drugs are widely used in clinics in the therapy of various disorders that resulted from atherosclerosis of different arteries. We attempted to uncover how calcium antagonists, beta-blockers and nitrates affect atherosclerotic indices of arterial cells.

We examined the effects of calcium antagonists on major atherosclerotic indices. We found that the calcium antagonist verapamil had a positive effect on all

atherosclerotic cellular indices. Within 48 hours, verapamil added to culture reduced the total intracellular cholesterol level 3-fold, sharply decreased the incorporation of [^{3}H]thymidine into cellular DNA (that is, suppressed cell proliferative activity), and inhibited the synthesis of collagen by cultured cells [101, 102]. Thus, this drug has a direct anti-atherosclerotic effect at the arterial cell level.

Several calcium antagonists, including nifedipine, darodipine, isradipine, nicardipine, nitrendipine, felodipine, tiapamil, gallopamil, diltiazem, papaverine, and nicardipine, were also tested. Verapamil and nifedipine proved to be the most effective [97, 103]. Within 24 hours of incubation with cultured cells, all calcium antagonists substantially inhibited [^{3}H]thymidine incorporation and reduced intracellular cholesterol levels [102, 103]. Thus, calcium antagonists produce a direct anti-atherosclerotic effect on the vascular cells, normalizing the major atherosclerotic cell parameters.

In addition to anti-atherosclerotic effects that induce the regression of atherosclerosis, the anti-atherogenic effects in culture that induce the prevention of atherosclerosis were also studied. Table 2 demonstrates the major differences between these two approaches. In the case of anti-atherosclerotic effects, the regression of atherosclerosis is induced, whereas in the case of anti-atherogenic effects, its prevention is induced. In the first case, the cells obtained from an atherosclerotic plaque are used, while in the second type of experiments, the cells are derived from an unaffected intima. When the anti-atherosclerotic effect is examined, cells are cultured in the presence of standard fetal calf serum, while experiments on anti-atherogenic effects utilize atherogenic sera obtained from coronary heart disease patients. These sera induce the accumulation of cholesterol and stimulate other atherogenic manifestations in cultured cells [109-112]. In the case of anti-atherosclerotic effects, the efficacy of a drug is judged by its ability to decrease an elevated content of cholesterol in cultured atherosclerotic cells. However, in the case of anti-atherogenic effects, drug efficacy is judged by the ability to prevent the deposition of intracellular cholesterol in normal cells.

Table **2** Anti-atherosclerotic and anti-atherogenic drug effects in culture.

ANTI-ATHEROSCLEROTIC	ANTI-ATHEROGENIC
Regression	Prevention
Atherosclerotic plaque	Uninvolved intima
Standard (nonatherogenic) serum	Atherogenic patients' serum
Cholesterol fall	Prevention of cholesterol accumulation

A four-hour preincubation of cultured cells with verapamil led to the complete prevention of the serum atherogenic effect [113]. Thus, verapamil possesses not only an atherosclerotic effect in culture, causing the regression of atherosclerotic manifestations at the cellular level, but it also demonstrates an anti-atherogenic, i.e., preventive, effect, eliminating the atherogenic potential of the serum.

The effect of several calcium antagonists on primary cholesterol accumulation in cultured cells induced by the patients' sera was tested. Verapamil and nifedipine completely inhibited the accumulation of intracellular cholesterol induced by the sera, while other calcium antagonists, such as diltiazem, nicardipine, isradipine, and darodipine, substantially reduced cholesterol accumulation [113]. The examined calcium antagonists demonstrated anti-atherogenic action in vivo by inhibiting the development of experimental atherosclerosis in animals [114, 115]. Thus, our in vitro data obtained using the cellular model correspond to the in vivo observations. One can conclude that calcium antagonists possess not only anti-atherosclerotic but also anti-atherogenic (preventive) effects at the arterial cell level.

Nitrates and beta-blockers have been tested to examine their effect on atherosclerotic cellular indices. Nitrates had no effect on the proliferative activity of atherosclerotic cells and only minimally affected cholesterol levels [103]. In contrast, all

the examined beta-blockers, i.e., propranolol, alprenolol, metoprolol, pindolol, and timolol, increased atherosclerotic manifestations, i.e., all of these drugs exhibited atherogenic activity in culture [103, 113]. If beta-blockers have a similar effect in vivo, one may assume that these drugs are atherogenic and induce their atherogenic effects at the arterial cell level. Apparently, nitrates do not follow a similar trend.

The influence of cardiovascular drugs on the atherosclerosis-related effects of each other has been studied [103, 113]. The study was focused on metoprolol, nifedipine, and nitroglycerin, drugs that are widely used in clinic. Metoprolol caused an elevation of intracellular cholesterol, and nifedipine reduced the cholesterol level. Furthermore, nitroglycerin did not have an effect on this index. The use of nifedipine in a background of metoprolol did not modify the anti-atherosclerotic action of the calcium antagonist. In this combination, the atherogenic action of metoprolol was not revealed. The application of metoprolol in combination with nitroglycerin led to the elimination of the atherogenic effect of the beta-blocker. Nifedipine used together with metoprolol and nitroglycerin was just as effective as in the absence of these drugs. Thus, nifedipine produces its anti-atherosclerotic effects both by itself and in combination with widely used nitrates and beta-blockers. These data suggest one important conclusion: the atherogenic action of beta-blockers can be inhibited if a beta-blocker is used in combination with a calcium antagonist or nitrate. This finding allows us to hope that it will be possible to develop beta-blockers that are devoid of atherogenic side effects.

Thus, three classes of cardiovascular drugs exert a different influence on the cellular manifestation of atherosclerosis. Calcium antagonists exhibit anti-atherosclerotic actions. In contrast, beta-blockers are atherogenic. Nitrates do not have an effect in this context. Our data obtained using the cellular model are consistent with the results of a clinical study. Loaldi et al. [116] reported that long-term perioral administration of propranolol aggravates coronary atherosclerosis in patients with angina of effort compared with the calcium antagonists nifedipiene and isorobide dinitrate. Nifedipine produced the best effect on coronary atherosclerosis by suppressing the development of

existing atherosclerotic lesions and preventing the appearance of new lesions. Isosorbide dinitrate was less effective in this respect, while the situation was the worst with propranolol therapy. These clinical observations encourage us to develop an anti-atherosclerotic therapy using our cellular model.

2.5. Ex Vivo Model

All of the above conclusions and hypotheses are based on the data obtained in in vitro experiments. Naturally, the question arises whether the anti-atherosclerotic effects of calcium antagonists and the atherogenic effects of beta-blockers can be manifested in vivo, and what is the optimal anti-atherosclerotic therapy based on calcium antagonists and other drugs?

To optimize anti-atherosclerotic and anti-atherogenic drug therapies, an ex vivo model was developed. In the ex vivo model, instead of drugs, blood sera taken from patients after oral drug administration is added to cultured cells.

The calcium antagonists verapamil and nifedipin and the beta-blockers propranolol and pindolol were examined using the ex vivo model [103, 104]. Within 2-4 hours after nifedipine or verapamil administration, the patients' sera had anti-atherosclerotic properties, i.e., the sera reduced the intracellular cholesterol and inhibited atherosclerotic cell proliferation. In contrast, the sera of patients who received propranolol or pindolol were pro-atherogenic. Its pro-atherogenic properties manifested themselves at the arterial cell level via the rise of intracellular cholesterol and the stimulation of cell proliferation. This finding allows us to assume that not only in vitro, but also in vivo, calcium antagonists and beta-blockers are anti-atherosclerotic and atherogenic drugs, respectively.

The effect of nifedipine on serum properties during a prolonged course has been assessed [113]. A patient was on nifedipine for 7 days. He received 20-mg doses three times a day at an 8-hour interval. Twenty-eight days after regular nifedipine therapy, the

initial atherogenicity of the patient's serum was substantially lower than at the beginning of the therapy. Directly after a dose of nifedipine, the atherogenicity was almost completely eliminated. In contrast, because of a prolonged therapy with the beta-blocker propranolol, the patient's serum acquired stable atherogenic properties. At the beginning of the course, the serum of this patient was nonatherogenic; however, 28 days of regular propranolol therapy led to the emergence of atherogenicity, revealed even before the drug administration. Thus, a single dose of beta-blockers induces temporary atherogenicity of serum. Prolonged therapy with beta-blockers leads to the emergence of stable atherogenic properties of patients' blood sera.

Table **3** Cholesterol content and proliferative activity of atherosclerotic cells cultured with lovastatin.

Drug concentration, M	Total cholesterol content, μg/mg cell protein	[^{3}H]Thymidine incorporation, dpm/μg cell protein
0 (control)	102±7	31±2
10^{-6}	105±9	29±2
10^{-5}	107±8	30±2
10^{-4}	100±10	35±4

Cells cultured from human atherosclerotic plaque were incubated during 24 hours with lovastatin, then atherosclerotic parameters were measured.

2.6. Indirect Anti-Atherogenic Effect of Lovastatin

The above-mentioned observations exemplify the use of cultured human aortic cells in an in vitro model for a mass screening of potential drugs and the investigation of their mechanisms of action. Cell culture can be employed in an ex vivo model to examine an indirect anti-atherogenic action of a drug and to optimize anti-atherosclerotic (anti-atherogenic) drug therapies. Using in vitro and ex vivo models, we have investigated the atherosclerosis-related effects of lovastatin.

The lipid-lowering drug lovastatin produced no effect on the atherosclerotic parameters of cultured human aortic cells isolated from an atherosclerotic lesion (Table 3), implying that the drug has no direct anti-atherosclerotic activity.

After a 6-month therapy with lovastatin (40 mg daily), the total blood cholesterol and LDL cholesterol levels decreased by 25 and 34%, respectively. These decreases were accompanied by a lowering of the cholesterol content of circulating immune complexes (Table 4), which reduced the atherogenic potential of the patients' sera. Sera obtained after treatment with lovastatin inhibited the accumulation of intracellular cholesterol compared with that induced by the sera before the therapy (Table 4). In some cases, lovastatin treatment completely eliminated serum atherogenic potential.

Thus, lovastatin possesses no direct effect on the atherosclerotic parameters of arterial cells; however, it exhibits indirect anti-atherogenic activity. This indirect action is realized as a reduction in the blood LDL level, which provokes a decrease in the LDL content of circulating immune complexes. Immune complexes with lower LDL content induce a lower accumulation of cholesterol in arterial cells, which should be considered as an indirect anti-atherogenic action of lovastatin.

Table **4** Effect of lovastatin treatment on cholesterol and low density lipoprotein levels, cholesterol content in circulating immune complexes and atherogenic potential of patients' blood serum.

Serum, #	Before treatment				After treatment			
	Total CH	LDL CH	CIC CH	Cell CH	Total CH	LDL CH	CIC CH	Cell CH
1	319	253	44.9	57±16	234	159	15.9	27±4
2	377	296	33.6	49±4	262	168	13.6	28±6
3	365	272	42.5	47±3	259	177	14.7	32±4
4	274	202	49.1	56±5	214	122	16.1	30±5
5	322	256	40.3	41±1	239	154	11.7	26±1
6	274	210	46.3	47±5	195	121	16.2	32±6
7	283	217	42.1	49±5	208	126	14.1	35±4
8	342	261	43.2	53±5	241	163	13.7	27±2

Patients were on lovastatin (40 mg/day) during 180 days. Values are expressed as mean of 4 determinations (± SEM). All differences between "before treatment" and "after treatment" are significant ($p<0.05$).

Total CH, total cholesterol content in blood serum (mg/dl); LDL CH, low density lipoprotein content (mg/dl); CIC CH, cholesterol content in circulating immune complexes (μg/ml); cell CH, total cholesterol content in normal aortic cells cultured 24 hours with patients serum (μm/mg cell protein). Cholesterol content in control cells cultured with fetal calf serum was 22±3 μg/mg cell protein.

2.7. In Vivo Model

In vivo models are necessary to confirm the results obtained on in vitro and ex vivo models and for developing new approaches to anti-atherosclerotic drug therapy.

We believe that some results obtained using our cellular model are applicable to clinical practice. To confirm these data by in vivo observations, we used rabbits with local lesions in the aorta induced by balloon catheter injury. This in vivo model is widely used as a simple approach to imitate human fibrous atherosclerotic lesions [117].

Using this model, we attempted to confirm the atherogenicity of beta-blockers and examine the possibility of eliminating this atherogenicity by combining a beta-blocker with a calcium antagonist. First, we showed that the oral administration of beta-blockers induces atherogenicity in rabbit blood serum, as also occurs in human patients. After oral administration of propranolol, methoprolol, athenolol, pindolol, or thimolol, blood was taken from the rabbit, and the blood serum was added to a culture of mouse peritoneal macrophages. All sera taken after beta-blockers considerably stimulated intracellular cholesterol accumulation, indicating that all these agents induce the atherogenic potential in the blood of rabbits (Table 5). For a detailed investigation of the atherogenicity provoked by beta-blockers, propranolol was chosen. This choice was motivated by its potent stimulating effect on intracellular cholesterol accumulation (Table 5).

After oral propranolol administration, the atherogenicity was manifested in 1 hour, reached the maximum in 2 hours, and remained elevated for at least 4 hours. Propranolol was given 3 times at 4-hour intervals. Second and third doses induced some increase in blood atherogenicity, which was elevated after the first dose. Twenty-four hours after the first dose followed by two additional doses, atherogenicity did not disappear and was maintained at an essentially high level; new doses produced only a slight increase in atherogenicity.

Table **5** Cholesterol accumulation in mouse peritoneal macrophages cultured with blood serum of rabbits treated with beta-blockers.

Drug	Dose, mg	Cholesterol accumulation, % of control
None (control)	-	100
Propranolol	20	195±5*
Metoprolol	50	126±9*
Atenolol	100	161±10*
Pindolol	5	129±8*
Timolol	2.5	194±19*

Data are expressed as mean of 3 determinations ± SEM.

*, significant difference from control ($p<0.05$).

Blood was drawn 2 hours after oral drug administration and blood serum was added to cultured mouse peritoneal macrophages. Four hours later cell cholesterol content was measured.

In our study, rabbits received propranolol three times a day for a 21-day period. This dosage imitated the 3-week course of a beta-blocker therapy. The blood serum became atherogenic after the first dose. Furthermore, the atherogenicity remained high throughout the 21-day period and was not elevated significantly by subsequent doses. At the end of the experiment, the animals were sacrificed, and the aorta was excised. On average, the neoinitma of propranolol-treated rabbits was 2-fold thicker compared with that of the controls (Table 6). Thus, oral propranolol administration evokes atherogenicity in the blood sera of rabbits. As in the case of atherosclerotic patients, prolonged treatment with this drug causes stable atherogenicity. In rabbits,

atherogenicity is accompanied by the stimulation of neointimal growth in the injured aorta. Therefore, the in vitro observations of propranolol atherogenicity in human cell culture and the ex vivo findings in a human model were confirmed by an animal model in vivo.

Table **6** Influence of papaverine on propranolol atherogenicity (% of control).

Parameter	Propranolol, 20 mg/day	Papaverine, 20 mg/day	Propranolol + papaverine
Intima : media ratio	177±4*	96±8	94±8
Cell number	305±9*	116±4	127±8
Cholesteryl esters	573±135*	107±11	94±16
Free cholesterol	244±32*	124±13	95±10
Triglycerides	355±31*	161±38	158±26
Collagen	172±20*	104±8	99±10

*, significant difference from control ($p<0.05$).

To confirm another very important result obtained on our models, namely, the ability of calcium antagonists to eliminate the atherogenic potential of beta-blockers, we examined a propranolol-papaverine combination. Papaverine was chosen because it exhibits a moderate anti-atherosclerotic activity [97]. Therefore, this drug can be regarded as a neutral or weak supplement to the beta-blocker.

Simultaneous administration of propranolol and papaverine eliminated the beta-blocker-induced blood atherogenicity. In contrast to the blood sera of rabbits given only propranolol, the sera of rabbits treated by a propranolol-papaverine combination

exhibited no increase in intracellular cholesterol. Throughout the 21-day period of propranolol-papaverine therapy, the rabbit blood sera displayed no atherogenicity, while the sera of propranolol-treated rabbits exhibited considerable atherogenic potential. Papaverine completely eliminated the stimulatory effect of propranolol on neointima formation (Table 6). In addition, the effects of propranolol and a propranolol-papaverine combination on other atherosclerotic indices of the rabbit aorta were studied. Propranolol increased the number of cells in the intima and stimulated the accumulation of triglycerides and esterified and free cholesterol, as well as the collagen production in the injury zone (Table 6). Papaverine produced no effect on these parameters (Table 6). In combination with papaverine, propranolol lost its atherogenic potential (Table 6). This phenomenon is not a result of the simple addition of the propranolol atherogenic effect to papaverine anti-atherogenic actions because papaverine by itself does not prevent the development of atherosclerosis in the injured rabbit aorta.

Thus, we have shown that papaverine eliminates the atherogenic side effect of propranolol. These results confirm our findings obtained in human models in vitro and ex vivo. We hope that the combination of a beta-blocker and an agent that eliminates its atherogenic potential will be helpful in the development of new drugs without atherogenic side effects.

2.8. Optimization of Dietary Therapy

The cellular model can be used not only to test drugs but can also be used to test foodstuffs as well. We have investigated the anti-atherosclerotic (therapeutic, causing regression of atherosclerosis) and anti-atherogenic (preventive) activities of certain mushroom species and sea products.

We have shown that alcohol and water extracts from 20 Korean mushroom species exhibit anti-atherosclerotic and anti-atherogenic activities in cell culture [107]. Thirteen of the 20 extracts tested were anti-atherosclerotic in culture, i.e., they caused a

decrease in the cellular cholesterol and/or inhibited the proliferation of atherosclerotic cells. Ten of 20 tested extracts displayed anti-atherogenic activity in addition to anti-atherosclerotic effects. Four mushroom species were chosen for the study of anti-atherosclerotic effects ex vivo. The cultivation of atherosclerotic cells during 24 hours in the presence of sera from healthy subjects who had had mushroom meals resulted in a 21-30% decrease in the cellular cholesterol level, that is, caused an anti-atherosclerotic effect [107]. The atherogenic sera obtained from atherosclerotic patients after dietary mushroom consumption partly (30-41%) lost its ability to increase the cellular cholesterol content [107]. Thus, the tested mushrooms exhibited anti-atherosclerotic and anti-atherogenic effects in an ex vivo model.

Among sea products, mollusk and krill meat have been tested. Specifically, patients were given canned meat of a mollusk belonging to the genus Buccinum. Two hours after a single dietary load, the patient's blood serum acquired marked anti-atherosclerotic properties (Table 7). The addition of this serum to cultured atherosclerotic cells led to a fall in intracellular cholesterol levels. Four hours later, the anti-atherosclerotic properties of the serum became even more prominent (Table 7).

Table **7** Effects of sea products on atherosclerosis-related properties of patients' plasma.

Product	Intracellular cholesterol content, μg/mg cell protein			
	Control	0 hours	2 hours	4 hours
Buccinum	196+17	204+16	181+19	150+16*
Krill	37+5	86+4	71+7	49+4*

*, Significant difference from 0 hr ($p<0.05$).

Patients of another group had initially atherogenic sera, causing more than a 2-fold increase in cholesterol content of cells derived from normal intima (Table 7). These patients received a single dietary dose of Antarctic krill meat. Two hours later, the atherogenicity of their blood sera decreased, and four hours later, it was practically absent (Table 7). Thus, krill meat exhibits a preventive anti-atherogenic action on arterial cells.

The results obtained suggest that the krill meat can be employed in diets aimed at the prevention of atherosclerosis. To develop a dietary therapy based on the krill meat, the effective dose and proper regimen should be established. As the first step to develop a dietary therapy, the following study was undertaken to determine the effective dose.

The patients' blood sera were analyzed for atherogenicity. Patients whose blood sera had an atherogenic potential were included in the study. The blood was collected from each patient before and 2 and 4 hours after a dose of krill meat. This protocol was repeated the next day with another dose of krill meat. Blood serum samples were added to a culture of subendothelial cells isolated from uninvolved human aortic intima, and intracellular cholesterol accumulation was assessed in each case. The anti-atherogenic activity of krill meat was evaluated by the ability to reduce serum atherogenicity, which was manifested in cholesterol accumulation in cultured cells. The dose-effect dependence was revealed by comparing the efficacy of the two doses. The efficacy of each dose was evaluated by the analysis of at least 6 sera obtained from different patients. It was observed that krill meat possesses anti-atherogenic effects at a dose of 10-20 g, half-maximum effect was reached at a dose of 30 g, and the maximum effect was achieved at a dose of 50 g.

We believe that this approach will be useful in the development and optimization of anti-aterhosclerotic and anti-atherogenic dietary therapies.

Table **8** Anti-atherogenic effects of natural products.

	Atherogenicity decrease, %
Spirulina platensis	51
Allium cepa	21
Beta vulgaris	31
Triticum vulgaris	70
Glycyrrhiza glabra	55
Salsola collina	11
Allium sativum	**77**
Pinus sylvestris	52

Chapter 3.
Natural Products for Anti-Atherosclerotic Therapy

The anti-atherogenic effects of dietary products promote the development of anti-atherosclerotic therapies based on natural products. Atherosclerosis develops over many years, so anti-atherosclerotic therapies should be long-term or even lifelong. For such long-term therapies, conventional medicine will not work. Drugs based on natural products can be a good alternative.

We have tested numerous natural products' extracts to reveal their effects on blood atherogenicity or the their capacity to prevent intracellular cholesterol accumulation caused by atherogenic blood sera from patients. Table 8 presents only the effective natural products. Naturally, the tested agents included anti-atherogenic, pro-atherogenic, and neutral products. Among the anti-atherogenic natural products, the most effective was garlic.

3.1. Mechanisms of Garlic's Anti-Atherosclerosis Effect

We extended the investigation of the in vitro effect of garlic extract on lipids of cultured human aortic cells. We previously showed that lipid accumulation in human aortic cells is accompanied by the stimulation of other cellular manifestations of atherosclerosis, namely, proliferation and extracellular matrix synthesis [89, 111]. Thus, the investigation of garlic's effect on cellular lipid parameters is closely related to the study of the mechanisms of garlic's anti-atherosclerosis effects.

Smooth muscle cells cultured from grossly normal intima and atherosclerotic plaque differed markedly in their contents of major lipid classes. Phospholipids, free cholesterol, cholesteryl esters, and triglycerides were much higher in atherosclerotic cells compared to cells derived from normal intima (Table 9). The addition of sera from patients with coronary heart disease to a culture of normal cells induced an intracellular

accumulation of all lipid classes except phospholipids (Table 9). After 24 hours of incubation with atherogenic serum, the content of cholesteryl esters increased 3.2-fold, and the of triglycerides and free cholesterol contents increased 1.7-fold, which amounted to 60-80% of the corresponding lipid contents of cells cultured from atherosclerotic plaques (Table 9). The addition of garlic prevented the serum-induced accumulation of free cholesterol and triglycerides and reduced the accumulation of cholesteryl esters 2.8-fold (Table 9). Additionally, in cells isolated from plaque, garlic lowered the levels of triglycerides, free cholesterol, and cholesteryl esters by 21%, 31%, and 35%, respectively (Table 9). Garlic had no significant effect on the total phospholipid content of smooth muscle cells cultured from grossly normal intima or atherosclerotic plaque (Table 9). In addition, it did not change the contents of sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine in either normal or atherosclerotic cells.

LDL is the main source of free and esterified cholesterol that accumulates in arterial cells. Arterial smooth muscle cells can bind, internalize, and metabolize LDL. The intracellular metabolism of LDL-derived cholesteryl esters was studied using lipoprotein labeled with [^{3}H]cholesteryl linoleate. The major proportion of LDL-derived cholesteryl esters was degraded to free cholesterol and reappeared in the culture medium (Table 10).

The LDL circulating in patients with coronary atherosclerosis is a modified lipoprotein. In contrast to native LDL of healthy subjects, the modified LDL induces the intracellular accumulation of cholesteryl esters. The uptake and degradation of cholesteryl esters supplied to the cell by the modified LDL was considerably greater than that of cholesteryl esters supplied by native LDL (Table 10). Garlic reduced the internalization of cholesteryl esters supplied by native and modified LDLs by 32% and 56%, respectively (Table 10). As a result, the intracellular accumulation of cholesteryl esters caused by native and modified LDLs was lowered almost 2- and 4-fold, respectively (Table 10).

Table **9** Effect of garlic powder extract on lipid composition in human aortic intimal smooth muscle cells.

	Lipid content, μg/mg cell protein			
	Phospholipids	Triglycerides	Free cholesterol	Cholesteryl esters
Normal cells				
Control	85.8±3.9	10.5+0.5	7.8+0.7	10.4+0.3
Atherogenic serum	92.3+6.4	17.5+0.7[a]	12.9+0.7[a]	33.1+1.2[a]
+ GPE, 1 mg/mL	84.4+3.0	12.8+0.4[b]	8.3+0.3[b]	18.4+0.7[ab]
Atherosclerotic cells				
Control	136.1+8.3	24.6+1.2	16.0+0.6	56.0+3.3
GPE, 1 mg/mL	131.8+8.0	17.0+1.0[a]	12.7+0.7[a]	34.2+1.5[a]

Data represent mean of three determinations + SEM from three separate experiments. Normal smooth muscle cells derived from grossly uninvolved intima and atherosclerotic cells derived from atherosclerotic plaque cultured in standard conditions served as the control. Atherogenic blood serum pooled from patients with coronary atherosclerosis was added to cultured normal cells to induce intracellular lipid accumulation. Cells with all additives were incubated during 24 hours.

GPE, garlic powder extract

a, Significant difference from the control, $p<0.05$.

b, Significant difference from atherogenic serum, $p<0.05$.

Thus, garlic suppresses LDL uptake and the intracellular degradation of LDL-derived cholesteryl esters, leading to a decrease in the intracellular accumulation of free and esterified cholesterol in arterial smooth muscle cells.

Table **10** Effect of garlic powder extract on the metabolism of low density lipoprotein derived cholesteryl esters in intimal smooth muscle cells.

	dpm/μg cell protein		
	Intracellular		Extracellular
	Cholesteryl esters	Free cholesterol	Free cholesterol
Native LDL	92+4	62+3	4872+119
Native LDL + GPE	49+3*	47+2*	3344+203*
Modified LDL	194+14	83+5	7400+503
Modified LDL + GPE	55+6*	60+4*	3295+289*

Smooth muscle cells cultured from uninvolved intima were incubated for 24 hours with native and modified (desialylated) low density lipoproteins (100 μg/mL) labeled with [^{3}H]cholesteryl linoleate.

GPE, garlic powder extract

*, Significant effect of GPE, $p<0.05$. For other details, see Table 9.

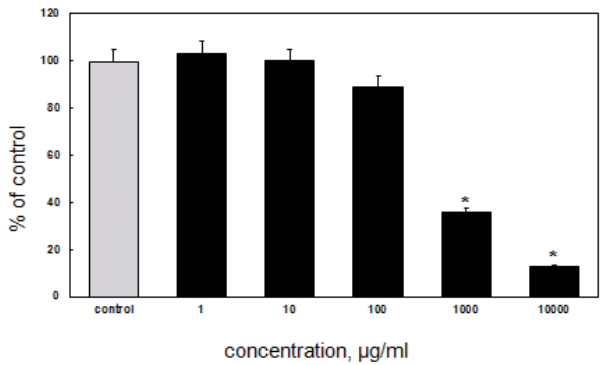

Figure **11** Effect of garlic powder extract on acyl-CoA:cholesterol acyltransferase (ACAT) activity in aortic smooth muscle cells cultured from atherosclerotic lesions. Each data point represents the mean of three determinations ± SEM. Significant differences ($p<0.05$) from the control (0 µg/mL garlic powder extract) are marked by an asterisk.

The effect of garlic on the enzymes responsible for the intracellular metabolism of cholesteryl esters was studied by measuring the activities of acyl-CoA:cholesterol acyltransferase (ACAT), the enzyme involved in cholesteryl ester formation, and cholesteryl ester hydrolase (CEH), which hydrolyzes cholesteryl esters. Enzyme activities were determined in homogenates of normal and atherosclerotic cells. The ACAT activity of atherosclerotic cells was 3-fold higher than that of normal cells. At concentrations of 0.1 mg/mL and greater, garlic powder extract significantly decreased the enzyme activity in homogenates prepared from both atherosclerotic and normal cells (Fig. 11). The CEH activity of atherosclerotic cells was almost 2-fold higher than that of

normal cells. At a concentration range of 0.01-1.0 mg/mL, garlic powder extract significantly increased the enzyme activity of both normal and atherosclerotic cells (Fig. 12). The dose-dependent effect of garlic had a bell-shaped pattern. The maximum effect was attained at 0.1 mg/mL; at higher and lower concentrations, the effect was less pronounced (Fig. 12). Thus, garlic inhibits ACAT, which participates in cholesteryl ester formation, and stimulates CEH, which degrades cholesteryl esters.

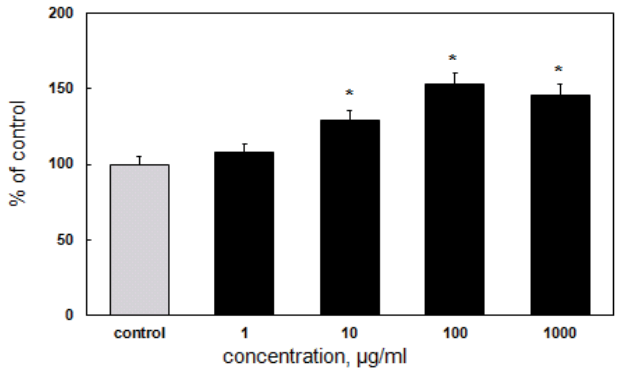

Figure **12** Effect of garlic powder extract on cholesteryl ester hydrolase (CEH) activity in aortic smooth muscle cells cultured from atherosclerotic lesion
Details are the same as in the legends to Figure 11.

Further investigations of garlic's anti-atherosclerotic effects included an ex vivo study and an animal model study. Both types of studies confirmed the in vitro effects of garlic [118]. Finally, we developed a drug based on garlic powder and carried out a clinical study of the effects of this drug on atherosclerosis regression.

3.2. Allicor (garlic)

As described above, garlic possesses direct anti-atherogenic effects, preventing cholesterol retention in arterial cells. We have developed and registered garlic powder tablets, currently produced by INAT-Farma, Ltd. (Russia). The AMAR study (Atherosclerosis Monitoring and Atherogenicity Reduction) was designed to estimate the effect of two-year treatment with the time-released garlic-based drug "Allicor" on the progression of carotid atherosclerosis in asymptomatic men in a double-blinded, placebo-controlled randomized clinical trial (ClinicalTrials.gov Identifier, NCT01734707). The primary outcome was the rate of atherosclerosis progression, measured by high-resolution B-mode ultrasonography as the increase in CIMT of the far wall of common carotid arteries [119].

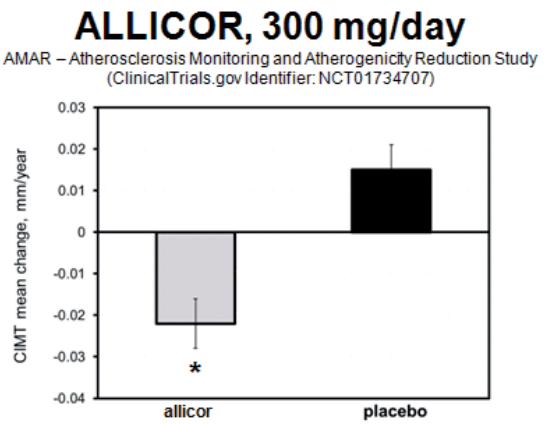

Figure **13** Anti-atherosclerotic effects of allicor.
Asterisks show a significant difference from the placebo group.

Allicor reduced CIMT compared to baseline and the placebo group. The difference in the CIMT changes between the allicor and placebo recipients was statistically significant (Pearson's chi-square 9.788, P=0.020). Thus, while spontaneous atherosclerosis progression prevailed in the placebo group, allicor beneficially affected early carotid atherosclerosis, significantly increasing lesion regression and reducing the net number of progressive lesions. The trend toward CIMT reduction in allicor recipients was observed after first 3 months of the study, and the CIMT measures were significantly different from the baseline measures and from the placebo group after the first 12 months of treatment [119]. At the end of the two-year study, the difference between the placebo and allicor recipients increased and remained statistically significant. The overall lesion progression was clearly different in the treated and untreated groups (Fig. 13). CIMT rose 0.015±0.008 mm annually, above a mean baseline IMT of 0.931±0.009 mm, in the placebo group, and fell in the allicor-treated patients at a rate of -0.022±0.007 mm per year (P=0.002). Though the benefit of allicor was more pronounced in year 1 (-0.028±0.008 mm), the benefit remained statistically significant in year 2 (-0.016±0.007).

The results obtained in our study are generally consistent with the data from a double-blinded, placebo-controlled randomized study by Koscielny et al. [120]. It has been demonstrated that 4-year treatment with the garlic-based drug Kwai inhibited the increase in volume of atherosclerotic plaques in carotid and femoral arteries by 5-18%. The age-dependent analysis of the plaque volume showed an increase between 50 and 80 years that was diminished under garlic treatment by 6-13% every 4 years. Therefore, with garlic application, the plaque volume in the whole collective remained practically constant within the age-span of 50-80 years [120].

The decrease in CIMT that was achieved during the AMAR study is comparable with the results of most successful trials with other compounds [121-126] (Table 11). However, these studies employed potent lipid-lowering agents or calcium antagonists, whose beneficial effects of treatment were attributed to reduction in LDL cholesterol,

the major risk factor for atherosclerosis development, or arterial wall stress.

Table **11** The comparative data from clinical trials on carotid atherosclerosis regression.

Trial	Medication	Mean annual IMT change, mm		Reference
		placebo	treatment	
PLAC II	Pravastatin	0.068	0.059	Crouse J.R. et al., 1995 [121]
KAPS	Pravastatin	0.029	0.010	Salonen R. et al., 1995 [122]
ASAP	Simvastatin	-	-0.009	Smilde T.J. et al., 2001 [34]
PREVENT	Amlodipine	0.011	-0.015	Pitt B. et al., 2000 [123]
ASAP	Atorvastatin	-	-0.020	Smilde T.J. et al., 2001 [34]
CLAS	Cholestipol, niacin	0.010	-0.020	Blankenhorn D.H. et al., 1993 [124]; Hodis H.N., 1995 [125]
MARS	Lovastatin	0.015	-0.028	Blankenhorn D.H. et al., 1993[126]; Hodis H.N., 1995 [125]
VHAS	Verapamil	-	-0.028	Zanchetti A. et al., 1998 [46]
AMAR	Allicor	0.015	-0.022	Orekhov et al., 2012 [119]

A direct influence of garlic on atherosclerosis has been discussed [127-131]. The anti-atherosclerotic effect of garlic has been attributed to its hypolipidemic activity. Experimental and clinical data have clearly demonstrated that garlic reduces blood cholesterol and LDL levels [131, 132]. The cholesterol lowering effect of garlic results from the inhibition of hepatic hydroxymethylglutaryl coenzyme A (HMG-CoA) activity [133]. In contrast to these studies, rather than examining the hypolipidemic effects of garlic, we examined the direct anti-atherosclerotic and anti-atherogenic effects of garlic, that is, the ability of garlic to act directly on the atherosclerotic process in the vessel wall. To investigate anti-atherosclerotic-related (therapeutic) effects, we used smooth muscle cells cultured from atherosclerotic plaques of the human aorta. To study anti-atherogenic-related (preventive) effects, we imitated atherogenesis in primary cultures of smooth muscle cells derived from grossly uninvolved human aortic intima by adding atherogenic blood sera of patients with angiographically assessed coronary atherosclerosis. Garlic decreased the triglyceride, cholesteryl ester, and free cholesterol contents of cells cultured from atherosclerotic plaques and prevented the atherogenic serum-induced accumulation of these lipids in cells cultured from grossly normal aorta. In other words, garlic possessed direct anti-atherosclerotic-related (therapeutic) and anti-atherogenic-related (preventive) effects. Garlic inhibits ACAT and stimulates CEH, thus displaying a direct influence on the synthesis and degradation of cholesteryl esters in the cell. This finding may explain the direct anti-atherosclerotic effects of garlic.

On the whole, the results of our study demonstrate that long-term treatment with the time-released, garlic-based drug allicor provides a direct anti-atherosclerotic effect on carotid atherosclerosis. As it is a remedy of natural origin, allicor is safe with respect to adverse effects and allows even perpetual administration, which may be quite necessary for the prevention and treatment of subclinical atherosclerosis. These results promoted the start of clinical trials of two other drugs based on natural products: Inflaminat, which possesses anti-cytokine activity, and the phytoestrogen-rich drug Karinat, which is designed for postmenopausal women.

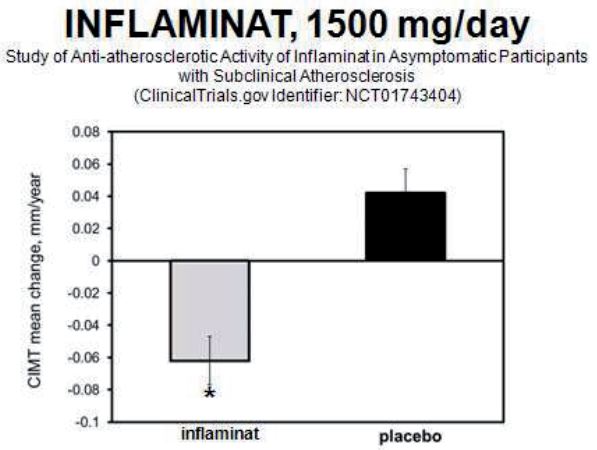

Figure **14** Anti-atherosclerotic effect of inflaminat.

3.3. Inflaminat (Calendula, Elder, Violet)

Atherosclerosis is regarded as a pathological process with elements of local aseptic inflammation, and inflammatory cytokines play a role at every stage of atherosclerosis development [134-136]. In this regard, drugs with systemic anti-inflammatory action may be effective for the prevention of atherosclerosis. The use of natural drugs is suitable for the early prevention of atherosclerosis because they have almost no side effects and exert regulatory effects at physiological limits, allowing longer, almost lifelong, medication. In this study, we investigated the atherosclerosis regression effect of the natural drug “Inflaminat”, which is based on calendula, elder and violet. These plants are widely used in herbal medicine as anti-inflammatory agents. In a pilot study (ClinicalTrials.gov Identifier, NCT01743404) of inflaminat using a protocol

similar to that of the AMAR study, inflaminat demonstrated atherosclerosis regression effects and a statistically significant difference from the baseline as well as from placebo group [119]. Figure 14 demonstrates the atherosclerosis regression effect of Inflaminat in asymptomatic men.

3.4. Karinat (Phytoestrogen-Rich Combination)

Atherosclerosis prevention in postmenopausal women is a striking problem because modern medicine does not provide an effective approach. Hormone replacement therapy has been rejected as a tool for atherosclerosis prevention in women due to the negative results of clinical studies, including WHI, PEPI, and HERS [137-142]. Therefore, the development of novel approaches is highly necessary. Phytoestrogens are often regarded as a possible alternative to hormone replacement therapy, but practically nothing is known about their effects on atherosclerosis.

We screened many natural phytoestrogen-rich components for their antiatherogenic activities using the above described ex vivo test system. The most promising of these compounds were garlic powder, extract of grape seeds, green tea leaves, and hop cones, all of which produced significant anti-atherogenic effects. Based on their combination, the novel isoflavonoid-rich dietary supplement “Karinat” was developed. It produces an efficient anti-atherogenic effect in cell culture models and is characterized by an improved phytoestrogen profile, providing additional amounts of biologically active polyphenols, including resveratrol, genisteine, and daidzeine, which are claimed to exert effects on atherosclerosis development. Karinat also contains additional amounts of β-carotene, α-tocopherol and ascorbic acid to provide the necessary daily intake of antioxidants.

Randomized, double-blinded, placebo-controlled pilot clinical studies on the anti-atherosclerotic effects of Karinat were performed in healthy peri- and postmenopausal women to characterize the risks and benefits of phytoestrogen therapy in relation to

atherosclerosis progression (ClinicalTrials.gov Identifiers, NCT01741974 and NCT01742000). The primary endpoint was the annual rate of changes in common carotid artery intima-media thickening, and the secondary endpoint was the dynamics of climacteric syndrome, which is monitored only in perimenopausal women. Figure 15 demonstrates the effect of karinat treatment on the dynamics of carotid atherosclerosis in postmenopausal women. In the placebo group, an increase in the average IMT of more than 100 μm per year was observed. Thus, the rate atherosclerosis in postmenopausal women was extremely high: the average increase in IMT was 13% per year, and the growth of atherosclerotic plaques was 40% per year. In the karinat group, a completely different picture was observed. The average CIMT was not changed (statistically insignificant increase of 6 μm per year, less than 1%). However, the progression of existing plaques by 27% per year was detected.

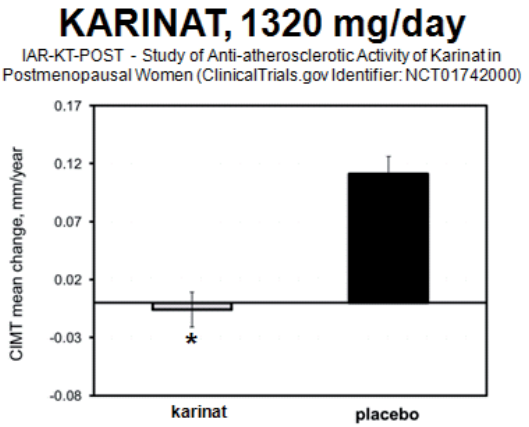

Figure **15** Anti-atherosclerotic effect of karinat.

The results of quantitative measurements of the degree of atherosclerosis in dynamics have shown that the use of the phytoestrogen complex in postmenopausal women almost completely suppresses the formation of new atherosclerotic lesions, and it slows the progression of existing lesions 1.5-fold [119].

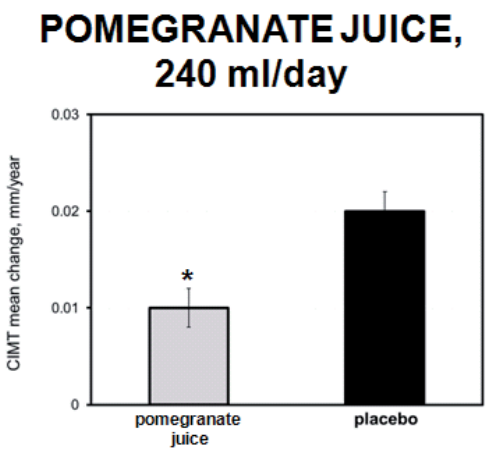

Figure **16** Anti-atherosclerotic effect of pomegranate juice.

3.5. Pomegranate Juice

A randomized, double-blind, parallel trial assessed the influence of the consumption of pomegranate juice on CIMT progression rates in subjects with a moderate risk of coronary heart disease [143]. Several CIMT-related parameters were measured; only one parameter among them demonstrated a significant anti-

atherosclerotic effect compared to placebo (Fig. 16). In general, the study showed no significant influence of pomegranate juice consumption on CIMT progression. However, the results from post hoc exploratory analyses suggest that the rate of CIMT progression may have been slowed in subgroups characterized by more rapid CIMT progression, including those with increased levels of triglyceride-rich lipoproteins, low levels of HDL cholesterol, and greater oxidative stress [144].

Conclusion

The results of numerous clinical trials with statins and other drugs have demonstrated the possibility of the prevention and regression of atherosclerosis by pharmacotherapy.

This review illustrates the use of cultured human arterial cells for the following:

1) the mass screening of drugs and chemicals (cyclic AMP elevators, calcium antagonists, prostaglandins, - blockers, antioxidants);

2) the investigation of the mechanisms responsible for the atherosclerosis-related effects (calcium antagonists and lovastatin);

3) the optimization of anti-atherosclerotic and anti-atherogenic drug and dietary therapy (β-blockers, calcium antagonists, mushrooms, krill meat).

The in vitro and ex vivo cellular models can be employed to reveal and investigate the following:

- direct anti-atherosclerotic activity – the regression of atherosclerosis (calcium antagonists, prostaglandins, antioxidants, lipostabil, mushrooms, mollusk meat);
- direct anti-atherogenic activity - prevention of atherosclerosis (calcium antagonists, mushrooms, krill meat);
- indirect anti-atherogenic activity (lovastatin);
- atherogenic activity (β-blockers, thromboxane, phenothiazines).

Natural products can be considered to be promising drugs for anti-atherosclerotic therapy. Two-year treatment with allicor (garlic powder) has a direct anti-atherosclerotic effect on carotid atherosclerosis in asymptomatic men. Inflaminat (calendula, elder and violet), which possesses anti-cytokine activity, caused the regression of carotid atherosclerosis in a 1-year treatment of asymptomatic men. The phytoestrogen-rich drug Karinat (garlic powder, extract of grape seeds, green tea leaves, hop cones, β-carotene, α-tocopherol and ascorbic acid) prevented the development of carotid atherosclerosis in postmenopausal women.

Our basic studies have shown that cellular lipidosis is the principal event in the genesis of atherosclerotic lesions. Using cellular models and natural products, we have developed an approach to prevent lipid accumulation in arterial cells. This led to the regression of atherosclerosis and/or the prevention of its progression in patients. Therefore, our basic findings were successfully translated into clinical practice. Because of this translation, a novel approach to anti-atherosclerotic therapy was developed. Based on our knowledge, we developed drugs that possess direct anti-atherosclerotic activity. Our clinical trial confirmed the efficacy of both the novel approach and novel drugs.

Unfortunately, natural products that possess anti-atherosclerotic therapeutic potential are not prescribed by medical practitioners as anti-atherosclerotic agents. However, the potential of these substances allows us to consider them as mainline additional supplements or prescriptions [144].

Acknowledgements

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