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Elsibucol inhibits atherosclerosis following arterial injury: Multifunctional effects on cholesterol levels, oxidative stress and inflammation

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ABSTRACT

Background: Elsibucol is a metabolically stable derivative of probucol with antioxidant, anti-inflammatory and antiproliferative properties. Here we investigated the effect of elsibucol on the development of atherosclerosis following arterial injury in hypercholesterolemic rabbits. **Methods and results**: New Zealand White rabbits were fed a high cholesterol diet that was supplemented or not with 0.5% elsibucol, 1% elsibucol or 1% probucol. An angioplasty of the iliac artery was performed after 3 weeks of diet. We found that treatment with elsibucol significantly decreases blood total cholesterol, LDLc and triglyceride levels. This is associated with a significant 46% reduction of neointimal hyperplasia following arterial injury. Interestingly, the effect of elsibucol on cholesterol levels and neointimal formation appears to be more pronounced than that of probucol. In vitro, elsibucol reduces vascular smooth muscle cell proliferation without affecting cell viability. In vivo, treatment with elsibucol is associated with a significant reduction of cellular proliferation (PCNA immunostaining), oxidative stress (nitrotyrosine immunostaining), VCAM-1 expression and macrophage infiltration in injured arteries. Despite its potent effect on neointimal hyperplasia, elsibucol does not prevent endothelial healing (Evans blue staining) following arterial injury. **Conclusions**: In hypercholesterolemic animals, elsibucol inhibits atherosclerosis and preserves endothelial healing following arterial injury. The mechanisms involved include lowering of blood cholesterol levels together with a reduction of oxidative stress and inflammation in injured arteries.

1. Introduction

Atherosclerosis is a complex pathology that involves several processes including subendothelial retention of atherogenic lipoproteins, oxidative stress, inflammation and cellular proliferation [1,2]. Therapies that are used clinically to inhibit atherosclerosis often target a single mechanism. For instance, compounds that prevent restenosis in coronary drug-eluting stents (DES) such as paclitaxel, sirolimus and sirolimus derivatives act through the inhibition of vascular smooth muscle cell (VSMC) proliferation [3]. However delayed endothelial healing [4], increased inflammation and oxidative stress [5], endothelial dysfunction [6] and local hypersensitivity responses with late coronary thrombosis [7] have all been described with these devices. Therefore, there is still an important clinical need to develop novel strategies that can target multiple components of the atherosclerotic process at the same time.

Probucol is a cholesterol-lowering drug with antioxidant and anti-inflammatory properties [8]. It has been shown to inhibit neointimal formation after balloon injury in swine coronary [9] and in rat and rabbit carotid arteries [10,11]. In humans, probucol can stop the progression of atherosclerotic plaques in carotid arteries [12] and inhibit restenosis in coronary arteries [13]. However, the long-term clinical use of probucol has been limited because it lowers HDL cholesterol (HDLc) and causes QTc interval prolongation on the electrocardiogram (ECG) [8,14]. Elsibucol (also known as AGI-1096) is a metabolically stable derivative of probucol [15]. It belongs to a class of new compounds that were designed to retain the antioxidant characteristics of probucol, without the negative effects on HDLc and QTc interval [16]. Elsibucol was initially developed for the prevention of organ transplant rejection. It was

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previously shown to have anti-inflammatory and antiproliferative properties in vitro, and to prevent graft vascular disease in a rodent aortic transplant model [15].

In the present study, we investigated the effect of elsibucol on atherosclerosis in a hypercholesterolemic rabbit model of arterial injury. Our results indicate that elsibucol inhibits atherosclerosis through multifunctional effects. Elsibucol lowers blood cholesterol levels and reduces cellular proliferation, oxidative stress and inflammation in atherosclerotic lesions. Importantly, we also demonstrate that elsibucol does not compromise endothelial healing following arterial injury.

2. Material and methods

2.1. Experimental animals

The protocol was approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the Centre Hospitalier de l'Université de Montréal (CHUM). New Zealand White male rabbits weighing 3.5–4.5 kg were obtained from Charles River Canada (St-Constant, QC). The animals received a 1% cholesterol diet (Harlan Teklad, Indianapolis, IN) for a total of 7 weeks. The diet was supplemented or not with 1% w/w probucol (Sigma, Oakville, ON, Canada); 0.5% w/w elsibucol (Astellas Pharma, Tokyo, Japan) or 1% w/w elsibucol. Each rabbit was given a 100 g portion of chow per day. An angioplasty of the iliac artery was performed after 3 weeks of diet. All animals received water ad libitum. For pharmacokinetic studies, blood samples were obtained 12–18 h after food withdrawal and shipped to Astellas Pharma (Northbrook, IL, USA) for the determination of elsibucol concentration levels. Plasma lipid and lipoprotein levels were determined by the biochemistry department of the CHUM.

2.2. Rabbit iliac balloon injury

Following premedication with acepromazine (1 mg/kg) and sedation with 5 mg/kg propofol, rabbits were intubated and anesthetised with 2% isoflurane in oxygen. A 5 Fr. introducer sheath (Terumo, Tokyo) was positioned in the carotid artery under surgical exposure. All catheters were subsequently introduced through this sheath. A Swan Ganz balloon catheter (Boston Scientific, Natick, MA) was then advanced over a 0.014″ guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, CA) into the left external iliac artery, inflated and retrieved from the iliac artery into the aorta as previously described [17]. This procedure was repeated three times to induce vascular injury.

2.3. Neointimal formation

Four weeks after balloon angioplasty, animals were sacrificed by barbituric overdose. A cannula was inserted into the lower abdominal aorta for *in situ* perfusion with a heparinized saline solution, followed by a 2–3 min perfusion with TissueFix. Sections of the iliac arteries were then stained with haematoxylin and eosin and intima/media ratios were determined using MetaMorph imaging system (Molecular Devices, Downingtown, PA) [17].

2.4. Reendothelialization

Reendothelialization was assessed by staining with Evans blue dye (Sigma), at 2 weeks after arterial injury as previously described [17]. Thirty minutes prior to sacrifice, 6 ml of 0.5% Evans blue dye was injected in the marginal vein of the ear. Animals were then sacrificed as described above; the iliac arteries were cut longitudinally and photographed. MetaMorph imaging system was used to measure and calculate the percentage of reendothelialization.

2.5. Immunohistochemical staining

Histological sections prepared from paraffin-embedded transverse cut of iliac arteries were used for immunoperoxidase and immunofluorescent analyses. The primary antibodies used were anti-nitrotyrosine (1:1000; Upstate, Billerica, MA), anti-rabbit macrophage (1:50; Dako, Glostrup, Denmark), anti-PCNA (1:200; Dako) and anti-VCAM-1 (1:200; Santa Cruz, Dallas, TX). Staining intensities were quantified in the neointima using ImageJ and the results were normalized by dividing the total intensities by the neointimal area. The results are reported in arbitrary units.

2.6. In vitro studies

Vascular smooth muscle cells (VSMC) were isolated from the thoracic aorta of healthy rabbits using an explant technique [18]. The cells were maintained in DMEM supplemented with 10% FBS, 200 units/ml penicillin and 0.25 mg/ml streptomycin. Cells were grown at 37 °C and 5% CO_2. For all experiments, early-passaged (P2 or P3) rabbit VSMCs were used. VSMCs were treated for 48 h with either 0.1% FBS (negative control) or 10% FBS and elsibucol 10, 12 or 15 μM. The concentrations of elsibucol were based on a previous report in vitro [15], and on preliminary dose–response studies indicating maximal biological activities at doses between 10 and 15 μM. VSMC proliferation was assessed using the MTS Celltiter 96 aqueous nonradioactive cell proliferation assay (Promega Madison, WI). VSMC viability was assessed by Hoescht 33342 and propidium iodide staining of unfixed/unpermeabilized cells.

2.7. Statistical analysis

Data are represented as the mean ± SEM. Groups were compared using a one-way ANOVA, followed by a Bonferroni post hoc test. A value of $p < 0.05$ was interpreted to denote statistical significance.

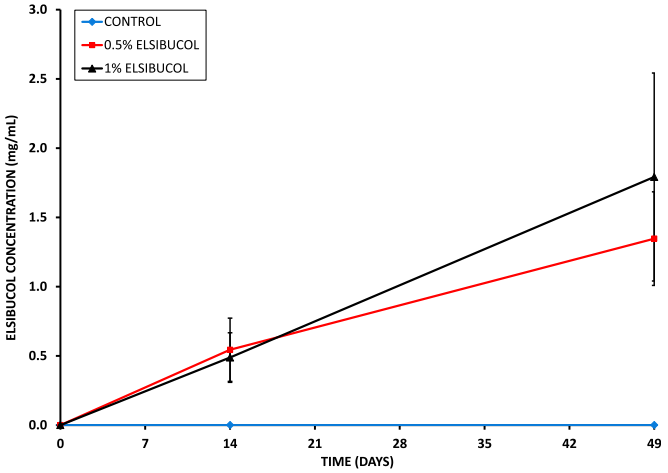

Fig. 1. Elsibucol plasma levels at different time points during the study. Rabbits were fed a high cholesterol diet only (control group in blue; $n = 4$) or also supplemented with either elsibucol 0.5% (red; $n = 4$) or elsibucol 1% (black; $n = 4$). Data are presented as mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)