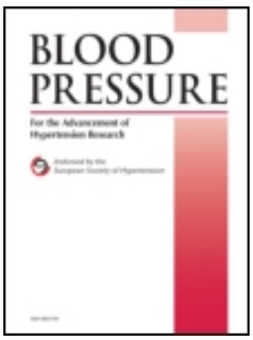

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The New Calcium Antagonist Lercanidipine and its Enantiomers Affect Major Processes of Atherogenesis *In Vitro*: Is Calcium Entry Involved?

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Corsini A, Accomazzo MR, Canavesis M, Sartani A, Testa R, Catapano AL, Fumagalli R, Paoletti R, Bernini F. *The new calcium antagonist lercanidipine and its enantiomers affect major processes of atherogenesis in vitro: is calcium entry involved?* Blood Pressure 1998; 7 (Suppl 2): 18–22.

Atherosclerosis results from multiple factors and involves several mechanisms, including endothelial monocyte and smooth muscle cell (SMC) changes, cholesterol accumulation, plaque rupture and thromboembolism. Calcium ions play a role in the initial and chronic development of atherosclerotic lesions. Several studies in experimental animal models have demonstrated the potential direct anti-atherosclerotic effects of calcium antagonists. In this study the antiatherogenic activity of lercanidipine, a new lipophilic, second-generation calcium antagonist, was investigated. Lercanidipine and its enantiomers inhibited the replication and migration of arterial myocytes in concentrations ranging from 10 to 50 μM. The antiproliferative effect of lercanidipine was dose dependent, with a potency similar to that of lacidipine and nifedipine, and was unrelated to the stereoselectivity of enantiomers to bind L-type calcium channels. Lercanidipine and its enantiomers (25 μM) decreased the serum-induced elevation of $[Ca^{2+}]i$ in SMC, with the (S)-enantiomer (69% inhibition) being 2.4-fold more active than the (R)-counterpart (29% inhibition). The studies performed with enantiomers of lercanidipine suggest that the observed effects are not related to the blockade of voltage-dependent Ca^{2+} channels and confirm, at least *in vitro*, the pharmacological potential of the compound to influence negatively the process of atherogenesis. *Key words: atherosclerosis, calcium antagonists, cytosolic calcium, Fluo 3, lercanidipine, L-type channels, smooth muscle cells.*

INTRODUCTION

The pharmacological treatment of atherosclerosis has greatly improved during the 1990s, owing to the introduction in therapy of new hypolipidaemic drugs such as the statins. Only about one-third of the treated individuals, however, benefits from this therapeutic regimen. It is, therefore, important to identify new agents that are active on different risk factors, or able to influence directly the atherogenic processes occurring in the arterial wall. As the migration and proliferation of smooth muscle cells (SMC) are mediated by calcium ions, in theory these processes may be affected by calcium antagonists (CA) [1, 2]. There is growing evidence that CA of different classes inhibit SMC migration and proliferation [1, 3, 4], an action that might explain part of their therapeutic value in atherosclerosis. The antiatherosclerotic effect of CA appears to be independent of serum lipid or blood pressure lowering [1, 5, 6] and thus provide an alternative approach to influencing the evolution of human atherosclerosis. Considering these findings, drugs able to control calcium homeostasis have received increasing attention as pharmacological tools for controlling abnormal myocyte migration and proliferation under atherogenic conditions [1, 4]. Animal models of vascular injury have shown that an arterial insult is followed by proliferation of the medial SMC, many of which migrate into the intima and proliferate further to form a neointimal lesion [7, 8]. The causes of these events are not completely understood. Recent findings have elucidated that SMC make up approximately 90–95% of the cellular population of the atherosclerotic lesion in young adults and compose an average of 50% of the advanced atherosclerotic plaque [9–11]. In addition, vascular myocytes contribute to the lesion by synthesis of the extracellular matrix and by their capacity to accumulate lipids and become foam cells [7, 9, 10, 12]. The present study investigated, using *in vitro* models, the effect of the new lipophilic dihydropyridine derivative lercanidipine [13, 14] and its enantiomers on arterial SMC migration and proliferation as related to L-type calcium-channel inhibition.

MATERIALS AND METHODS

Lercanidipine hydrochloride {methyl 1,1-dimethyl-2-[*N*-

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(3,3-diphenylpropyl)-*N*-methylamino] ethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate} and its enantiomers were dissolved in ethanol (1% final concentration) and control cell dishes received the same volume of the solvent. Nifedipine was from Sigma (St Louis, MO, USA), lacidipine from Glaxo (Verona, Italy), isradipine from Sandoz (Milan, Italy), diltiazem–HCl from Lusofarmaco (Milan, Italy) and verapamil–HCl (Isoptin) from Knoll (Liestal, Switzerland).

Eagle's minimum essential medium (MEM), fetal calf serum (FCS), trypsin-ethylenediaminetetracetic acid (EDTA), penicillin (10 000 U/ml), streptomycin (10 mg/ml), tricine buffer (1 M, pH 7.4) and non-essential amino acid solution (100 ×) were from Gibco (Madison, WS, USA). Disposable culture flasks and Petri dishes were from Corning Glassworks (Amedfield, MA, USA). [^{3}H]Nitrendipine s.a. 87 Ci/mmol was from NEN Research Product (Cologno Monzese, Italy). 6-[^{3}H]Thymidine s.a. 2 Ci/mmol was from Amersham (Amersham, UK) and Isoton II was purchased from Coulter Instruments (Milan, Italy). Fluo 3 was purchased from Molecular Probes (Eugene, OR, USA).

Nifedipine, lacidipine and verapamil were dissolved in ethanol, isradipine was solubilized in a water/ethanol mixture (70/30 v/v) and diltiazem–HCl was dissolved in distilled water before assay.

The affinity of lercanidipine and its enantiomers for the dihydropyridine subunit of the calcium channels was studied in rat brain membranes labelled by [^{3}H]nitrendipine according to the method published previously [14].

Smooth muscle cells were cultured, according to Ross [15], from the intima-media layer of the aorta of male Sprague–Dawley rats (200–250 g). Cells were grown in monolayers at 37°C in a humidified atmosphere of 5% CO2 in MEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 mM tricine buffer and 1% (v/v) non-essential amino acid solution [16]. Cells were used between the fourth and tenth passage. Human vascular myocytes (A 617 from human femoral artery) were grown in the same culture conditions [16, 17].

Migration of rat SMC was induced by fibrinogen and examined using a 48-well microchemotaxis chamber (Neuro-Probe, Cabin John, MD, USA). SMC that migrated to the lower surface of the filters were counted under a high-power (100×) field (HPF). Six HPF were counted per sample and the results were averaged [3].

To investigate the proliferation of SMC, cells were seeded at various densities for rat (2×10^5) and human (5×10^4) myocytes/Petri dish (dia. 35 mm) and incubated with MEM supplemented with 10% FCS [19]. Cell proliferation was evaluated by cell count after trypsinization of the monolayers using a Coulter Counter model ZM [16]. SMC doubling time was computed according to Elmore and Swift [18]. Cell proliferation was then estimated by nuclear incorporation of [^{3}H]thymidine. The amounts of drug required to inhibit 50% of cell proliferation and of cell migration were calculated by linear regression analysis of the logarithm of the concentration (μM) vs logit [19].

Measurement of the concentration of cytosolic Ca^{2+} was performed using the fluorescent probe Fluo 3 [20].

Table I. *Effect of calcium antagonists on [^{3}H]nitrendipine binding sites of rat brain membranes*

Drug	Inhibition (IC50 nM)
Nifedipine	3.52 ± 0.45
(RS)-Lercanidipine	0.59 ± 0.19
(R)-Lercanidipine	84.83 ± 31.69
(S)-Lercanidipine	0.27 ± 0.05

IC50 = concentrations of the drug required to inhibit the specific binding of [^{3}H]nitrendipine (0.5 nM) by 50%.
Data are given as mean ± SD of three experiments, each carried out in triplicate.

RESULTS

Lercanidipine and its (S)-enantiomer displaced [^{3}H]nitrendipine from rat brain membranes with higher potency than nifedipine (Table I). The affinity of the (R)-enantiomer for the 1,4-dihydropyridine binding sites was about 300-fold lower than that of the (S)-counterpart. The potential antiproliferative action of the dihydropyridine derivative lercanidipine was studied in rat aortic myocytes at drug concentrations ranging from 10 to 50 μM and compared with that of CA of various classes.

In a first set of experiments, the effect of the compound was evaluated by cell counting after 3 days of growth and exposure to the drug. Lercandipine decreased SMC proliferation in a concentration-dependent manner and treated cells had longer doubling times than those of controls (Table II). The ability of lercanidipine and its

Table II. *Effect of (RS)-lercanidipine on the doubling time for rat aortic myocytes*

Addition	Doubling time (h)
None	37.8 ± 2.1
10 μM	38.1 ± 0.9
25 μM	62 ± 5.6*
50 μM	121.9 ± 28.2**

The doubling time was measured after 72 h of incubation; Data are given as mean ± SD of three experiments.
* $p < 0.01$; ** $p < 0.001$ (Student's *t*-test): significant difference between drug and control groups.

enantiomers to inhibit SMC proliferation was unrelated to their potency to bind L-type calcium channels. The racemate and the two enantiomers were equipotent in inhibiting cell proliferation (Fig. 1).

When compared with other CA, lercanidipine appeared to be one of the more effective antiproliferative agents, with an IC50 (the concentration required to inhibit specific binding by 50%) value within the range of lacidipine and nifedipine (Table III). Similar results were obtained when investigating the effect of lercanidipine on the proliferation of human myocytes (IC50 = 22.5 μM).

When human myocytes were treated with the tested compounds for 72 h and the incubation medium was removed and replaced with fresh medium, cells were able to recover from the inhibitory effect, demonstrating that the inhibition of SMC proliferation by lercanidipine and its enantiomers was not the result of cytotoxicity.

In subsequent experiments, cellular growth was measured as nuclear incorporation of labelled thymidine by rat aortic myocytes and the (R)-enantiomer appeared to be the most effective form (Table IV).

The ability of lercanidipine and its enantiomers to interfere with the migration of arterial myocytes was studied in rat aortic myocytes incubated for 5 h in the presence of fibrinogen as a chemotactic factor [21, 22]. All of the tested compounds were able to inhibit myocyte migration in a dose-dependent manner, with the (R)-enantiomer showing the most pronounced effect (Table V).

FCS (10%) induced a rapid and transient increase in $[Ca^{2+}]i$, which attained levels four-fold higher than basal values in rat control cells. Preincubation of the cells with either (R)- or (S)-lercanidipine (25 μM) for 24 h induced a reduction in $[Ca^{2+}]i$ even after the removal of the drug from the incubation medium; inhibition was 29%

Table III. *Effect of calcium antagonists on the proliferation of rat aortic myocytes*

Drug	Inhibition (IC50 μM)
Nifedipine	34.3 (21.8–56.7)*
Lacidipine	27.6 (20.8–36.7)*
Isradipine	13.1 (10.3–16.8)*
(RS)-Lercanidipine	31.2 (27.2–36.6)*
(R)-Lercanidipine	30.7 (27.5–33.4)*
(S)-Lercanidipine	33.3 (30.9–34.8)*
Diltiazem	112.4 (78.8–160.5)*
Verapamil	53.2 (32.6–87.1)*

* 95% confidence limits.

Table IV. *Effect of lercanidipine and its enantiomers on [^{3}H]thymidine incorporation by rat aortic myocytes*

	Inhibition (IC50 μM)
(RS)-Lercanidipine	17.2 (13.8–22.1)*
(R)-Lercanidipine	11.6 (8.7–15.6)*
(S)-Lercanidipine	25.4 (21.6–30.8)*

* 95% confidential limits.

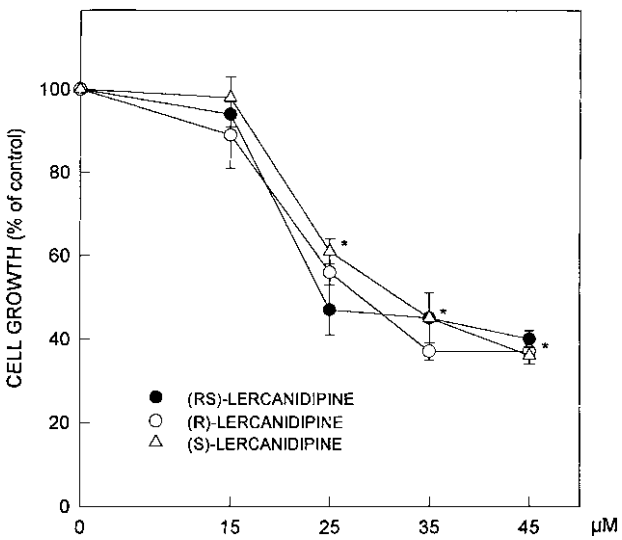

Fig. 1. Effect of lercanidipine and its enantiomers on proliferation of rat aortic myocytes. Cells were seeded at a density of 2×10^5/dish and incubated with a medium supplemented with 10% FCS; 24 h later the medium was changed for one containing 0.4% FCS to stop cell growth and the cultures were incubated for 72 h. At this time (time 0) the medium was replaced with one containing 10% FCS and the reported concentrations of the tested compounds. The incubation was continued for a further 72 h at 37°C. Each point represents the mean ± SD of triplicate dishes. The mean value of control (100%) was 1280×10^3 cells/plate ($\pm$ 28×10^3) $p < 0.0001$ (Student's *t*-test): significant difference between drug and control groups.

Table V. *Effect of calcium antagonists on the fibrinogen-induced migration of rat aortic myocytes*

Drug	Inhibition (IC50 μM)
Nifedipine	181 (41.5–774.7)*
Isradipine	n.a.
Diltiazem	n.a.
Verapamil	n.a.
(R,S)-Lercanidipine	21.4 (14.8–32.8)*
(S)-Lercanidipine	18.3 (11.6–30.8)*
(R)-Lercanidipine	6.0 (1.2–39.6)*
Lacidipine	12.3 (9.6–16.6)*

n.a. = not active. Isradipine was tested up to 20 μM, verapamil and diltiazem up to 100 μM.
* 95% confidential limits.

($p < 0.05$, $n = 8$) and 69% ($p < 0.001$, $n = 7$) for R and S enantiomers, respectively.

DISCUSSION

The present results indicate that lercanidipine is effective in reducing the proliferation and migration of arterial myocytes. The antiproliferative effect of the drug, evaluated as cell number and nuclear incorporation of thymidine, was dose dependent, with a potency similar to that obtained with lacidipine and nifedipine [3, 23]. Furthermore, when compared with other CA, lercanidipine appears to be one of the most potent antichemotactic agents, with an IC50 similar to that of lacidipine. Both new CA are clearly lipophilic compounds.

An important relationship has been demonstrated between cell growth and calcium ions [1, 24]. However, the mechanism by which CA affect SMC growth is unknown.

The present results show that the antiproliferative effect of lercanidipine and its enantiomers is unrelated to their potency as calcium-channel blockers, a result that would argue against the involvement of Ca^{2+} influx via L-type channels in their antiatherosclerotic activity. This conclusion is further supported by experiments showing that the serum-induced Ca^{2+} influx in SMC was strongly reduced by the (S)-enantiomer as expected; however, the (R)-enantiomer, displaying about 300-fold lower affinity for the L-type calcium-channel, was also able to decrease partially $[Ca^{2+}]i$, probably owing to the high concentration tested. The (R)-enantiomer, moreover, is slightly more potent in inhibiting SMC migration and DNA synthesis than its counterpart and the racemate.

The toxicity of lercanidipine and its enantiomers has been ruled out as being responsible for the antiproliferative effect. None of the compounds tested under the present experimental conditions was cytotoxic: the cells excluded trypan blue and started growing again after removal of the drugs.

The (R)-enantiomer of lercanidipine could represent a therapeutic hope as a direct antoatherosclerotic agent, practically devoid of antihypertensive activity [25]. Preliminary data obtained in the authors' laboratory indicate that lercanidipine and its enantiomers may also inhibit low-density lipoprotein oxidation and cholestrol esterification in cultured mouse peritoneal macrophages, suggesting a potential effect of these compounds on the lipid components of atheroma.

In summary, these data indicate that the new calcium antagonist lercanidipine and its enantiomers are able to inhibit SMC proliferation and migration in a dose-dependent manner. The ability of lercanidipine to interfere with these processes through a mechanism independent of calcium-channel blockade confers a pharmacological interest on the compound in the process of atherogenesis.

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