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Fluvastatin Synergistically Improves the Antiproliferative Effect of Everolimus on Rat Smooth Muscle Cells by Altering p27^{Kip1}/Cyclin E Expression

N. Ferri, A. Granata, C. Pirola, F. Torti, P. J. Pfister,[1] R. Dorent, and A. Corsini

Department of Pharmacological Sciences, University of Milan, Milan, Italy (N.F., A.G., C.P., F.T., A.C.); Novartis-Pharma AG, Basel, Switzerland (P.J.P.); and Department of Cardiology, Tenon Hospital, Assistance Publique, Hopitaux de Paris, Paris, France (R.D.)

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ABSTRACT

Multiple intracellular signaling pathways stimulate quiescent smooth muscle cells (SMCs) to exit from G_0 and re-enter the cell cycle. Thus, a combination of two drugs with different mechanisms of action may represent a suitable approach to control SMC proliferation, a prominent feature of in-stent restenosis. In the present study, we investigated the effect of everolimus, a mammalian target of rapamycin inhibitor, in combination with fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, on proliferation of rat SMCs. The antiproliferative action of everolimus was amplified by 2.5-fold by the addition of subliminal concentrations of fluvastatin (5×10^{-7} M), lowering the IC_{50} value from 2.5×10^{-9} to 1.0×10^{-9} M. The increased antiproliferative effect of everolimus by fluvastatin was prevented in the presence of mevalonate, farnesol, or geranylgeraniol, suggesting the involvement of prenylated proteins. Cell cycle analysis and [^{3}H]thymidine incorporation assay demonstrated that the two drugs synergistically interfered with the progression of G_1 phase. In particular, the drug combination significantly up-regulated p27^{Kip1} levels by 47.0%, suppressed cyclin E by 43.0%, and it reduced retinoblastoma (Rb) hyperphosphorylation by 79.0%, compared with everolimus alone. Retroviral overexpression of cyclin E conferred a significant resistance of rat SMCs to the antiproliferative action of the drug combination, measured by cell counting, [^{3}H]thymidine incorporation, and cell cycle analysis, with higher levels of hyperphosphorylated form of Rb. Taken together, these results demonstrated that everolimus acts synergistically with fluvastatin to inhibit SMC proliferation by altering the expression of cyclin E and p27^{kip1}, which affects Rb phosphorylation and leads to G_1 phase arrest.

Smooth muscle cell (SMC) proliferation in the arterial wall is the major determinant of restenosis after balloon angioplasty and stent coronary implantation (Ross, 1999; Hansson, 2005). The introduction of drug-eluting stent has significantly improved the restenosis process and the patient outcome after revascularization; but recently, the safety and the efficacy of this approach have been reevaluated (Boden et al., 2007; Stone et al., 2007). Thus, single and/or combined oral therapy has been proposed as promising approach to achieve a better clinical outcome after percutaneous coronary intervention (Mody et al., 2001; Boden et al., 2007). In particular, a combination of two different pharmacological inhibitors capable of antagonizing different intracellular signaling pathways involved in cell cycle reentry may lead to better control of SMC proliferation.

The 40-*O*-(2-hydroxyethyl)-derivative of rapamycin, everolimus, is a proliferation signal inhibitor that affects growth factor-induced proliferation of hematopoietic and nonhematopoietic cells via cell cycle arrest at the late G_1 phase (Price et al., 1992; Brown et al., 1995; Decker et al., 2003; Hafizi et al., 2004). The antiproliferative action of everolimus is elicited through binding to the mammalian target of rapamycin complex (mTORC) 1 composed of mTOR, a common regulatory subunit called LST8, and the raptor subunit that specifies the downstream substrates (Schuler et al., 1997; Sarbassov et al., 2004; Shaw and Cantley, 2006). The binding of everolimus to mTORC1 complex strongly inhibits its catalytic activity and the activation of two well characterized mTORC1 complex substrates that control translation and cell growth,

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[1] Current affiliation: Nicox S. A., Sophia Antiplois, France.

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ABBREVIATIONS: SMC, smooth muscle cell; mTOR, mammalian target of rapamycin; mTORC, mammalian target of rapamycin complex; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; FOH, farnesol; GGOH, geranylgeraniol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MVA, mevalonate; Rb, retinoblastoma; IRES, internal ribosomal entry site; cdk, cyclin-dependent kinase; SCH66336, lonafarnib.

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the p70S6 protein kinase (p70S6) and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) family of proteins (Brown et al., 1995; Brunn et al., 1997). More recently, everolimus has been shown to directly interfere with the assembly of the rapamycin-insensitive rictor/mTOR protein complex, mTORC2, and to block AKT signaling (Zeng et al., 2007). Thus, the inhibition of both mTORC1 and mTORC2 is considered the pivotal molecular mechanism for the antiproliferative effect of everolimus.

The inhibition of cell proliferation is thought to be the basic molecular mechanism for the multiple actions of everolimus, such as immunosuppression, prevention of renal and heart transplant rejection, and retardation of cardiac allograft vasculopathy (Schuler et al., 1997; Nashan, 2002). In an experimental model of in-stent restenosis, oral administration of everolimus inhibited SMC proliferation at similar degree to that seen with rapamycin-eluting stents, suggesting a potential oral use of this drug for restenosis (Farb et al., 2002). This feature has made rapamycin and everolimus an attractive pharmacological tool for the development of drug-eluting stents. Indeed, everolimus-eluting stents as rapamycin-eluting stents, have been reported to inhibit in-stent neointimal growth in patients with coronary artery disease (Grube et al., 2004).

A second class of drugs that strongly affects cell proliferation is represented by the HMG-CoA reductase inhibitors, also called statins. We have previously shown that fluvastatin interferes with SMC proliferation in vitro at therapeutic concentrations ($0.1–1 \times 10^{-6}$ M), and more importantly, sera from patients treated with fluvastatin can significantly reduce SMC proliferation in an ex vivo assay (Corsini et al., 1996). The ability of statins to inhibit SMC proliferation seems to be independent from their cholesterol-reducing properties, and more likely to be related to the depletion of intracellular nonsteroidal isoprenoid compounds, such as farnesol (FOH) and geranylgeraniol (GGOH), which inhibits intracellular protein prenylation process (Corsini et al., 1993; Raiteri et al., 1997; Bellosta et al., 2000). Several prenylated proteins belonging to different intracellular signaling pathways have been documented to be indispensable for cell proliferation, including the small GTP-binding protein Ras, and Ras-like proteins, such as Rho, Rac, and Rap (Corsini et al., 1999; Brown et al., 2006).

Interestingly, the combination fluvastatin everolimus has been shown previously to have a beneficial effect on graft vascular disease in a rat model of chronic heart rejection, measured as arterial intimal thickness, suggesting a potential positive effect between the two drugs on SMC proliferation. The basic molecular mechanisms, however, have not been elucidated (Gregory et al., 2001).

On this basis, in the present study we explored the potential synergistic inhibitory effect of the combination everolimus fluvastatin on SMC proliferation and the underling molecular mechanisms.

Materials and Methods

Reagents and Antibodies. DMEM, trypsin ethylenediaminetetraacetate, penicillin (10,000 U ml^{-1}), streptomycin (10 mg ml^{-1}), 1 M Tricine buffer, pH 7.4, nonessential amino acid solution (100×), and fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA). Disposable culture flasks and Petri dishes were from Corning Life Sciences (Acton, MA), and filters were from Millipore Corporation (Billerica, MA). [6-^{3}H]Thymidine, sodium salt (2 Ci/mmol) was from GE Healthcare (Milan, Italy), and molecular weight protein standards were from Bio-Rad Laboratories (Hercules, CA). Isoton II was purchased from Instrumentation Laboratories (Milan, Italy). SDS, TEMED, ammonium persulfate, glycine, and acrylamide solution (30% T, 2.6% C) were obtained from Bio-Rad Laboratories. Cytox-Dye was purchased from Invitrogen. Fluvastatin (Corsini et al., 1995) and everolimus (SDZ RAD) (Schuler et al., 1997) were provided by Novartis-Pharma AG (Basel, Switzerland). FOH, GGOH, and mevalonate (MVA) were from Sigma (Milan, Italy). For Western blot analysis, the following antibodies were used: anti-cyclin D1, anti-cyclin E, anti-cdk2, anti-p70S6 kinase, and anti-phospho-p70S6 kinase Thr 412 (Millipore, Vimodrone, Italy); anti-p27^{Kip1} (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Rb protein (Millipore); anti 4E-BP1 (Cell Signaling Technology Inc., Danvers, MA); anti-p21^{Cip1} (Abcam plc, Cambridge, UK); and anti-mouse and anti-rabbit peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

Cell Proliferation and DNA Synthesis. SMCs were cultured from the intimal-medial layers of aorta of male Sprague-Dawley rats as described previously (Corsini et al., 1995). Cells were seeded at a density of 1×10^5 SMC/35-mm Petri dish, and then they were incubated with DMEM supplemented with 10% FCS. Twenty-four hours later, the medium was changed to medium 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 medium containing 10% FCS in the presence or absence of known concentrations of the drugs, and the incubation was continued for further 72 h at 37°C. Cell proliferation was evaluated by cell counting with a Coulter Counter model ZM (Beckman Coulter, Fullerton, CA) after trypsinization of the monolayers. At time 0, just before the addition of the substances to be tested, three Petri dishes were used for cell counting. The total cell number determined at time 0 was subtracted from cell number found in each triplicate after 72 h of cell growth.

For DNA synthesis, synchronization of SMCs to the G_0/G_1 phase of the cell cycle was accomplished by incubating logarithmically growing cultures (3×10^5 myocytes/Petri dish) for 5 days in a medium containing 0.4% FCS. Quiescent cells were then incubated for 16 h in fresh medium containing 10% FCS in the presence or absence of drugs. DNA synthesis was estimated by nuclear incorporation of [^{3}H]thymidine (Ferri et al., 2003).

HMG-CoA Reductase Assay. The experimental conditions were the same than those used for cell proliferation assay. HMG-CoA reductase activity was determined by measuring the rate of conversion of radioactive HMG-CoA into MVA in detergent-solubilized cell-free extract (Corsini et al., 1995). Aliquots of the cell-free extracts (30–40 μg) were assayed in a buffer containing 0.25 M K_2HPO_4, pH 7.4, 100 mM glucose 6-phosphate, 15 mM NADP, 50 mM dithiothreitol, and 110 μM HMG-CoA (90,000 dpm/sample [^{14}C]HMG-CoA) in a total volume of 200 μl. Microsomes were preincubated in the reaction buffer at 37°C for 10 min before the addition of HMG-CoA, and then they were incubated for 120 min at 37°C with moderate shaking. The reaction was stopped by the addition of 20 μl of 5 M HCl, and 90,000 dpm [^{3}H]mevalonolactone standard was added to measure recovery. The reaction solution was then incubated at 37°C for 30 min to allow lactonization of the mevalonate. The mixture was extracted twice with 10 ml (20 ml total) of diethyl ether. The top phase was transferred to a 50-ml conical tube, and the combined upper phases were dried; the residue was resuspended in acetone, spotted on a thin layer chromatography plate, and chromatographed in acetone/benzene (1:1). The activity of HMG-CoA reductase was expressed as cpm incorporated in mevalonate per microgram of detergent-solubilized protein.

Cell Cycle Analysis. The experimental conditions used were the same as those used for DNA synthesis assay. Flow cytometry was used to analyze cell cycle distribution. Cells were trypsinized and centrifuged for 5 min at 1000 rpm. Pellets were resuspended in 0.5

ml of permeabilizing buffer of Cytox Dye (0.5 μM in 100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% Nonidet P-40). Samples were placed in the dark for 30 min, and the fluorescence of individual nuclei was measured. Nuclear Cytox Dye fluorescence signal was recorded on the FL2 channel of a FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed with ModFit LT software (Verity Software House, Topsham, ME). The number of cells in G_0/G1, S, and G_2/M phases was expressed as percentages of total events (10,000 cells) (Ferri et al., 2003).

Western Blot Analysis. Cells were washed twice with phosphate-buffered saline and lysed by incubation with a solution of 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% Nonidet P-40, containing protease and phosphatase inhibitor cocktails (Sigma) for 30 min on ice. Cell lysates were cleared by centrifugation at 14,000*g* for 10 min, and protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). Lysates were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, transferred to Immobilon polyvinylidene difluoride (Millipore Corporation), and subsequently immunoblotted with primary antibody following appropriate secondary antibody, before visualization by enhanced chemiluminescence (GE Healthcare). Quantitative densitometric analyses were performed using GelDoc acquisition system and Quantity One software (Bio-Rad Laboratories).

Generation of Cyclin E Expression Construct and Retroviral Infection. Full-length rat cyclin E (accession no. D14015) was generated by polymerase chain reaction using the following primers: 5′-ATGAAAGAAGAAGGTGGTTCCG-3′ and 5′-TCATTCTGTCTCCTGCTCACTGC-3′. The sequence of the polymerase chain reaction-generated construct was confirmed by sequencing. Retroviral expression plasmid was then constructed using the pBM-IRES-PURO (Garton et al., 2002) expressing the puromycin resistance gene as a selectable second cistron gene, generated from the original pBM-IRES-EGFP, generously provided by Garry P. Nolan (Stanford University, Stanford, CA). Retroviral infections of human SMC were performed as described previously (Garton et al., 2002).

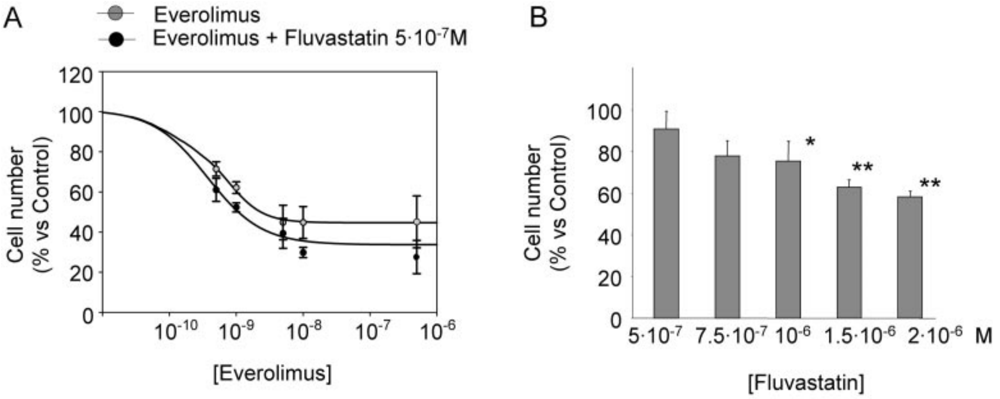
Analysis of Drug Synergism. According to the method of Kern et al. (1988), the expected value of cell number (CN_{exp}, defined as the product of the percentage versus control of cell number observed after incubation with drug A alone and the percentage of cell number observed for drug B alone divided by 100) and the actual cell number observed (CN_{obs}) for the combination of A and B were used to construct a synergistic ratio as follows: $R = CN_{exp}/CN_{obs}$. Synergy was defined as any value of R greater than unity. An R value of 1.0 (additive effect) or less indicated an absence of synergy (Kern et al., 1988).

Statistical Analysis. Experimental data are expressed as mean ± S.D. The effects of the tested drugs versus control on the different parameters were analyzed by two-tailed Student's *t* test for unpaired data. The concentration of everolimus required to inhibit 50% of cell proliferation (IC_{50}) was calculated by nonlinear regression curve (SigmaPlot software; Systat Software, Inc., Point Richmond, CA).

Results

Fluvastatin Synergistically Improves the Inhibitory Effect of Everolimus on Arterial SMC Proliferation. The antiproliferative effect of everolimus was studied on rat aortic SMCs at concentrations ranging from 5×10^{-10} to 5×10^{-7} M. As shown in Fig. 1A, everolimus decreased SMC proliferation in a concentration-dependent manner. The concentration of everolimus required to inhibit cell proliferation by 50% (IC_{50}) was 2.5×10^{-9} M. Although everolimus very potently reduced cell proliferation, it did not allow a complete inhibition with 54.9 ± 12.9% inhibitory effect at 5×10^{-7} M (Fig. 1A). A plateau of about 55% inhibition was reached at concentrations of 5×10^{-9} M and above (Fig. 1A).

The inhibitory effect of everolimus on SMC proliferation was then evaluated in combination with subliminal fluvastatin concentration (5×10^{-7} M). Fluvastatin alone resulted in a nonsignificant 9.18 ± 8.4% inhibitory effect on rat SMC proliferation (Fig. 1B). The combination with fluvastatin led to a potent inhibitory effect of everolimus on cell proliferation, with an IC_{50} value equal to 1.0×10^{-9} M, 2.5-fold lower than that observed with everolimus alone. The combination of fluvastatin with everolimus increased the extent of inhibition of cell proliferation from 54.9 ± 12.9% to a maximum



Everolimus (mol/L)	Cell number (% vs control) Everolimus		Everolimus + Fluvastatin		R (expected/observed)
	Everolimus alone	Fluvastatin ($5 \cdot 10^{-7}$ mol/L) alone	expected	observed	
$5 \cdot 10^{-10}$	71.5	89.8	64.1	61.1	1.05
10^{-9}	62.2		55.8	52.3	1.07
$5 \cdot 10^{-9}$	44.8		40.2	39.4	1.02
10^{-8}	44.8		40.2	29.8	1.35
$5 \cdot 10^{-7}$	45.1		40.5	27.6	1.47

Fig. 1. Effect of everolimus alone or in combination with fluvastatin on rat SMC proliferation. A, cells were seeded at a density of 1×10^5 per 35-mm dish and incubated with DMEM supplemented with 10% FCS; 24 h later, the medium was changed with medium containing 0.4% FCS to stop cell growth, and the cultures were incubated for 72 h. At this time, the medium was replaced with medium containing 10% FCS, in the presence or absence of indicated concentrations of drugs. After 72 h, at 37°C, cell number was evaluated by cell counting after trypsinization of the monolayers. Each bar represents the mean ± S.D. of six different experiments. B, experimental conditions are the same as described in A. Each bar represents the mean ± S.D. of three different experiments. *, $p < 0.05$ and **, $p < 0.001$, fluvastatin versus control (Student's *t* test). C, synergistic effect of the combination everolimus with fluvastatin is demonstrated by the R value greater than unity. The synergistic ratio represents the ratio of expected inhibitory effect on cell proliferation and the observed inhibition (Kern et al., 1988).

inhibitory effect of 72.4 ± 8.3% at 5×10^{-7} M everolimus concentration (Fig. 1A; Table 1).

To determine whether the antiproliferative action of fluvastatin in combination with everolimus was additive or synergistic, data were analyzed according to Kern et al. (1988). The combination of the two drugs produced an additive effect until 5×10^{-9} M and a synergistic effect with higher concentrations, with R values of 1.35 and 1.47 at 10^{-8} and 5×10^{-7} M, respectively (Fig. 1C).

In a first attempt to determine the molecular mechanism responsible for this synergistic action, we evaluated the effect of everolimus on the inhibitory activity of fluvastatin on its intracellular target (i.e., HMG-CoA reductase). As shown in Fig. 2A, 5×10^{-7} M fluvastatin up-regulated the HMG-CoA reductase by 52.5-fold, indicating a significant inhibition of the enzyme in SMCs (Corsini et al., 1995). A similar induction of HMG-CoA reductase was observed when fluvastatin was combined with 10^{-8} M everolimus (57.4-fold), indicating that the addition of everolimus did not alter the pharmacological action of fluvastatin.

The primary targets of mTORC1, inhibited by everolimus, are p70S6 kinase and 4E-BP1 (Fingar et al., 2004). The activation state of p70S6 kinase is closely related to the phosphorylation of threonine 412 residue, a modification that is often used as an in vivo readout of mTOR activity (Pearson et al., 1995). As shown in Fig. 2C, both everolimus alone (10^{-8} M) and in combination with fluvastatin (5×10^{-7} M) completely suppressed the threonine 412 phosphorylation of p70S6 kinase after 3 days of exposure. Moreover, everolimus alone and in combination with fluvastatin inhibited, at the same extent, the accumulation of the phosphorylated form of 4E-BP1 (Fig. 2C).

These results demonstrated that the combination of the two drugs did not significantly alter the inhibitory action of fluvastatin and everolimus on HMG-CoA reductase and mTOR, respectively.

Because the antiproliferative action of fluvastatin is dependent by the inhibition of protein prenylation through a reduced intracellular availability of FOH and GGOH (Corsini et al., 1993; Laufs et al., 1999), we investigated the preventing effect of MVA, the product of the HMG-CoA reductase, and FOH and GGOH, the substrates of protein prenyl transferases (Winter-Vann and Casey, 2005), on the antiproliferative effect of this drug combination. As shown in Fig. 2C, the coincubation with MVA, FOH, or GGOH abolished the synergistic antiproliferative effect of the combination everolimus fluvastatin. These data suggest that fluvastatin enhanced the inhibitory effect of everolimus on cell proliferation by affecting the synthesis of the MVA-derived isoprenoid, FOH and GGOH, substrates of protein prenyl transferases, and potentially by interfering with protein prenylation.

The Synergistic Effect of Combination Everolimus Fluvastatin Is Elicited in G_1 Phase. Everolimus has been reported to induce a cell cycle arrest at the G_1 phase by blocking mTOR activity (Schuler et al., 1997; Azzola et al., 2004), but the effect of the association with fluvastatin has not been studied. We therefore investigated the effect of everolimus alone and in combination with fluvastatin on cell cycle progression using two different approaches: [^{3}H]thymidine incorporation assay and flow cytometry analysis of the cell cycle. As shown in Fig. 3, everolimus inhibited, in a concentration dependent manner, the DNA synthesis induced by 10% FCS, with an IC_{50} value of 6.47×10^{-9} M. The combination of everolimus with 2×10^{-6} M fluvastatin, a concentration that did not significantly inhibit [^{3}H]thymidine incorporation, increased the potency of everolimus to block DNA synthesis, leading to an IC_{50} value of 1.9×10^{-10} M (Fig. 3A; Table 1).

We next studied the effect of everolimus in association with fluvastatin, on the progression of the cell cycle from G_1 to S phase by flow cytometry analysis. Incubation of SMCs with 0.4% FCS led to accumulation of cells at G_0/G_1 phase (94.8 ± 2.1%) with only a small percentage at S phase (2.1 ± 0.3%). After incubation with 10% FCS, we observed a significant increase in the proportion of SMCs in S phase (22.2 ± 3.3%), which was decreased to 5.1 ± 0.8% by 10^{-7} M everolimus (Fig. 4). It is noteworthy that we observed no significant increase in the percentage of cells at sub-G_0/G_1 phase, demonstrating a specific antiproliferative activity of everolimus without any induction of apoptosis (Fig. 4). Although everolimus almost completely inhibited the progression from G_1 phase to S phase, the combination with 2×10^{-6} M fluvastatin significantly reduced the percentage of cells at S phase to 3.7 ± 0.3% (Fig. 4; $P < 0{,}05$). Taken together, these results indicated that the synergistic antiproliferative action of everolimus in combination with fluvastatin may be elicited in G_1 phase of the cell cycle.

Fluvastatin Significantly Improves the Effect of Everolimus on Cyclin E Expression and Rb Phosphorylation. To better define the antiproliferative action of the combination fluvastatin everolimus in G_1 phase, we carried out a series of experiments aiming at evaluating the expression levels of cyclins expressed in the G_1/S phase transition, such as cyclin E and cyclin D1 (Adams, 2001). Western blot analysis of total cell lysates for cell cycle proteins showed that cyclin D1 and cyclin E were strongly induced after 16 h of the addition of 10% FCS to the culture medium, compared with the quiescent conditions containing 0.4% FCS (Fig. 5). As expected, the addition of subliminal concentrations of fluvastatin (2×10^{-6} M) that did not alter either [^{3}H]thymidine incorporation or cell cycle progression (Figs. 3 and 4) did not significantly change both cyclin D1 and E expression

TABLE 1

Inhibitory effect of everolimus alone or in association with fluvastatin on cell proliferation

Assay	Inhibition (Conc.) for Fluvastatin	IC_{50}		Ratio	Maximal Effect (Conc.)	
		Everolimus	Everolimus + Fluvastatin		Everolimus	Everolimus + Fluvastatin
		nM				
Cell proliferation	−9.2 ± 8.4% (0.5 μM)	2.5	1.0	2.5	−54.9 ± 12.9% (0.5 μM)	−72.4 ± 8.3% (0.5 μM)
Thymidine incorporation	−14.2 ± 9.1% (2 μM)	6.5	0.19	34.2	−63.3 ± 4.6% (0.1 μM)	−75.9 ± 2.4% (0.1 μM)
Cell cycle (S phase)	3.2 ± 23.8% (2 μM)				−76.9 ± 3.8% (0.1 μM)	−83.4 ± 1.5% (0.1 μM)

levels. However, 10^{-7} M everolimus reduced by 36.2 and 26.8% cyclin D1 and cyclin E levels, respectively (Fig. 5). The addition of fluvastatin further enhanced the effect elicited by everolimus, leading to 46.1 and 58.0% reduction of cyclin D1 and E, respectively. We next analyzed the expression levels of cdk2 and its inhibitors $p27^{Kip1}$ and $p21^{Cip1}$. Whereas $p27^{Kip1}$ was not altered by either fluvastatin or everolimus alone, the combination of everolimus with fluvastatin led a significant increase of its expression levels by 47.0% compared with everolimus alone (Fig. 5). In contrast, the addition of fluvastatin to everolimus did not alter the expression levels of both cdk2 and $p21^{Cip1}$ in cells incubated with everolimus alone. Nevertheless, everolimus alone significantly reduced the expression levels of $p21^{Cip1}$ induced by the addition of 10% FCS by 42% (Fig. 5).

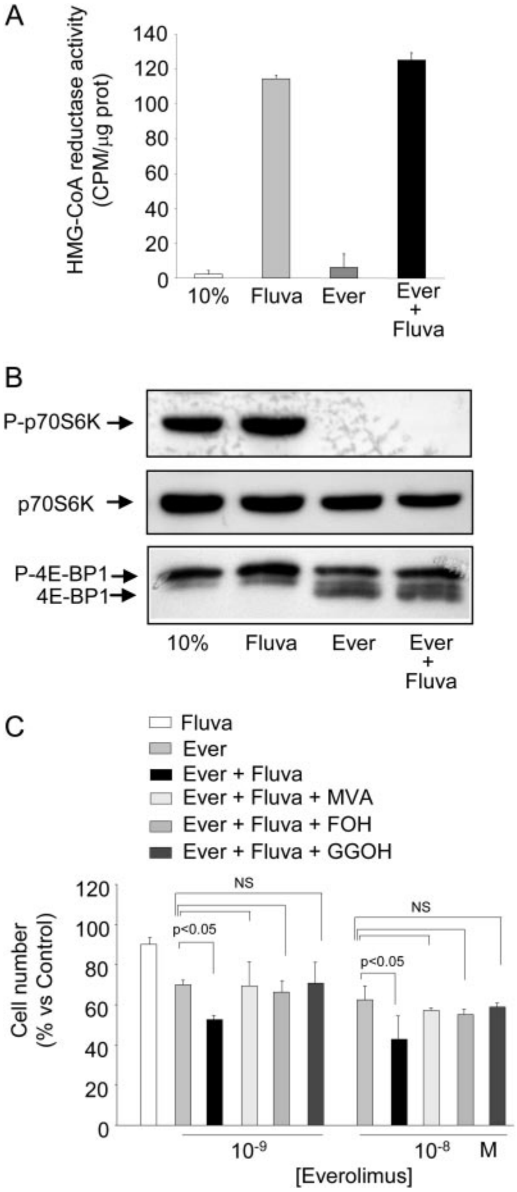


Fig. 2. Effect of everolimus alone or in combination with fluvastatin on HMG-CoA reductase activity, and p70S6 kinase and 4E-BP1 phosphorylation. A, synchronized rat SMCs were incubated for 72 h with DMEM containing 10% FCS in the presence or absence of fluvastatin (5×10^{-7} M), everolimus (10^{-8} M), and their combination. After this period, the HMG-CoA reductase activity was determined by measuring the rate of conversion of radioactive HMG-CoA into mevalonate in detergent-solubilized cell-free extract as described under *Materials and Methods*. Each bar represents the mean ± S.D. of triplicate samples. B, under the same experimental conditions described for A, p70S6 kinase (p70S6K) expression and phosphorylation on threonine 412 (P-p70S6K) was determined by Western blot analysis of total protein extracts using the polyclonal antibodies anti-p70S6 kinase and anti-phospho-p70S6 kinase. The same analysis was performed to evaluate the expression levels and the phosphorylation state for 4E-BP1. C, under the same experimental conditions, cell number was evaluated by cell counting after incubation with fluvastatin (5×10^{-7} M), everolimus (10^{-8} and 10^{-9} M), and their combination in the presence or absence of MVA (10^{-4} M), FOH (10^{-5} M), or GGOH (5×10^{-6}). Each bar represents the mean ± S.D. of two different experiments. Statistical analysis was performed by Student's *t* test. NS, not significant.

Finally, we analyzed the phosphorylation state of Rb protein by Western blot analysis. The addition of 10% FCS to the culture medium clearly induced Rb hyperphosphorylation, an event that was not affected by fluvastatin but significantly reduced by everolimus alone (−43.2%) (Fig. 5). The combination of everolimus and fluvastatin led to an almost complete inhibition of Rb hyperphosphorylation (−87.6%), a condition similar to that observed in quiescent cells (0.4% FCS), indicating a cell cycle arrest in G_1.

Altogether, the present results indicate that everolimus affected SMC proliferation by interfering with the progression of the G_1 phase, reducing the expression of both cyclin D1 and cyclin E and the phosphorylation of Rb protein. The combination with subliminal concentrations of fluvastatin resulted in a more significant inhibitory effect on SMC growth and the expression of cyclin E and Rb phosphorylation, with increased $p27^{Kip1}$ levels.

Overexpression of Cyclin E Confers a Partial Resistance to the Antiproliferative Action of Combination Everolimus Fluvastatin. To directly address the role of cdk2/cyclin E complex on the synergistic effect of the combination everolimus fluvastatin, cyclin E were overexpressed in rat SMCs. Western blot analysis of total cell lysates shows that exogenous cyclin E was efficiently overexpressed in rat SMCs compared with cells transduced with PURO control vector (data not shown). These established cell lines were then used for determining the antiproliferative action of the combination fluvastatin everolimus. After 3 days, the combination of the two drugs (fluvastatin 5×10^{-7} M and everolimus 10^{-7} M) led to 87.9 ± 2.0% inhibition of cell proliferation in control SMCs and 67.7 ± 3.7% in cells overexpressing cyclin E (Fig. 6A). A partial resistance was also observed by evaluating the S phase entry determined by [^{3}H]thymidine incorporation assay and flow cytometry analysis after 16 h of incubation with fluvastatin at 2×10^{-6} M and everolimus at 10^{-7} M (Fig. 6, B–D). Indeed, [^{3}H]thymidine incorporation in control and cyclin E-overexpressing cells was equal to 16.3 ± 0.4 and 24.9 ± 4.8% versus control, respectively (Fig. 6B). Quantification of the percentage of cells in S phase by cell cycle analysis showed that, after 16 h of exposure to the drug combination, a significant lower number of control cells was replicating the DNA (8.64 ± 0.6%) compared with cyclin E-overexpressing cells (11.1 ± 1.1%). The resistance of cells overexpressing cyclin E was also confirmed by the presence of higher hyperphosphorylated form of Rb after 16 h of exposure to the drug combination compared with control cells (Fig. 6D).

Taken together, the forced overexpression of cyclin E had a slight, but significant impact on the antiproliferative action of the combination everolimus fluvastatin, indicating that cyclin E down-regulation is required for a full exploitation of the inhibition of cell growth by these two drugs.

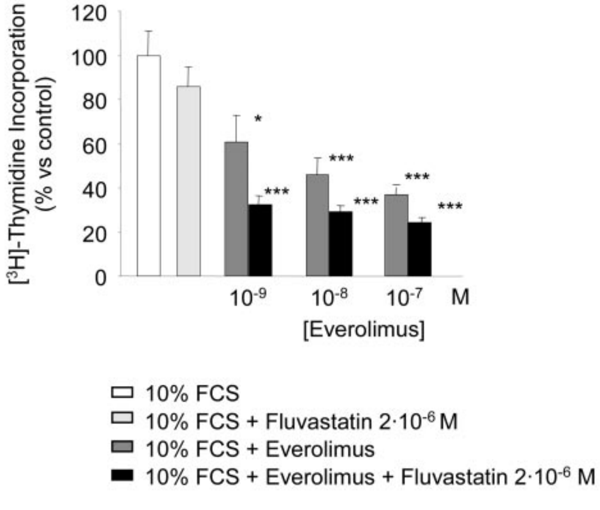
Discussion

The Combination of Subliminal Concentrations of Fluvastatin Synergistically Improve the Antiproliferative Action of Everolimus. The present study was undertaken to explore the antiproliferative action of everolimus on rat aortic SMC proliferation alone and in combination with fluvastatin. The present findings demonstrated, for the first time, a synergistic antiproliferative effect between fluvastatin and everolimus measured by cell counting after 3 days of exposure to the drugs and by $[^3H]$thymidine incorporation assay after 16 h of incubation (Table 1). Moreover, by biological and pharmacological approaches and genetic modification of rat SMCs, we demonstrated that the synergistic effect of this drug combination converges on the regulation of cyclinE/p27^{kip1} complex, leading to a block in G_1 phase of the cell cycle.

The IC_{50} values, a measure of pharmacological potency, showed that everolimus plus fluvastatin was, respectively, 2.5- and 32.7-fold more potent than everolimus alone to affect cell proliferation and $[^3H]$thymidine incorporation, respectively (Table 1). In terms of efficacy, everolimus led to a growth inhibition up to 54.9 ± 12.9%, an effect that was enhanced to 72.4 ± 8.3% by the combination with subliminal concentration of fluvastatin (Table 1). Similar plateau of about 55% inhibition was also observed in previous studies conducted with everolimus in tumor cell lines, and association with other chemotherapeutic agents led to a more profound inhibitory activity (Haritunians et al., 2007). The analysis of drug interaction clearly demonstrated that the antiproliferative effect of everolimus in the presence of a subliminal concentration of fluvastatin was synergistic at concentration of everolimus higher than 5×10^{-9} M

The Synergistic Antiproliferative Effect of Everolimus Fluvastatin Is Elicited in G_1 Phase of the Cell Cycle by Affecting p27^{kip1}/Cyclin E Expression. Because flow cytometry analysis of the cell cycle supports the possibility that fluvastatin enhances the inhibitory effect of everolimus at the level of the progression of G_1/S phase, we studied the expression levels of G_1 phase proteins by Western blot analysis.

The current knowledge of the molecular mechanism for cell cycle entry of eukaryotic cells emphasized a pivotal role for cdk4/cdk6 and cdk2 and their respective cyclin partners cyclins D1, D2, D3, E, and A (Adams, 2001). In particular, the expression of D type cyclins seems to be controlled by the extracellular mitogens, and, once induced, D type cyclins associate with cdk4 and cdk6, mediating the phosphorylation of Rb protein. In contrast, the expression of E type cyclins is controlled by an autonomous mechanism and peaks sharply at the G1/S border (Ekholm and Reed, 2000; Aleem et al., 2005), and they are believed to complete the phosphorylation of pRb, initiated by the action of cyclin D-cdk complexes (Stacey, 2003; Fu et al., 2004). More recently, the generation of cyclin E knockout mice allowed for the demonstration of their essential function in cell cycle re-entry (Geng et al., 2003). In our study, the antiproliferative effect of the tested drugs was assessed in SMCs previously synchronized in G_0 phase of the cell cycle and then stimulated by the addition of 10% FCS. Under these experimental conditions, the up-regulation of cyclin E is absolutely required for cell cycle progression (Geng et al., 2003). Consistently with previous studies conducted with rapamycin in SMCs (Braun-Dullaeus et al., 2001), everolimus did not affect p27^{kip1} expression levels in SMCs, but a significant up-regulation (+47.0%) was ob-



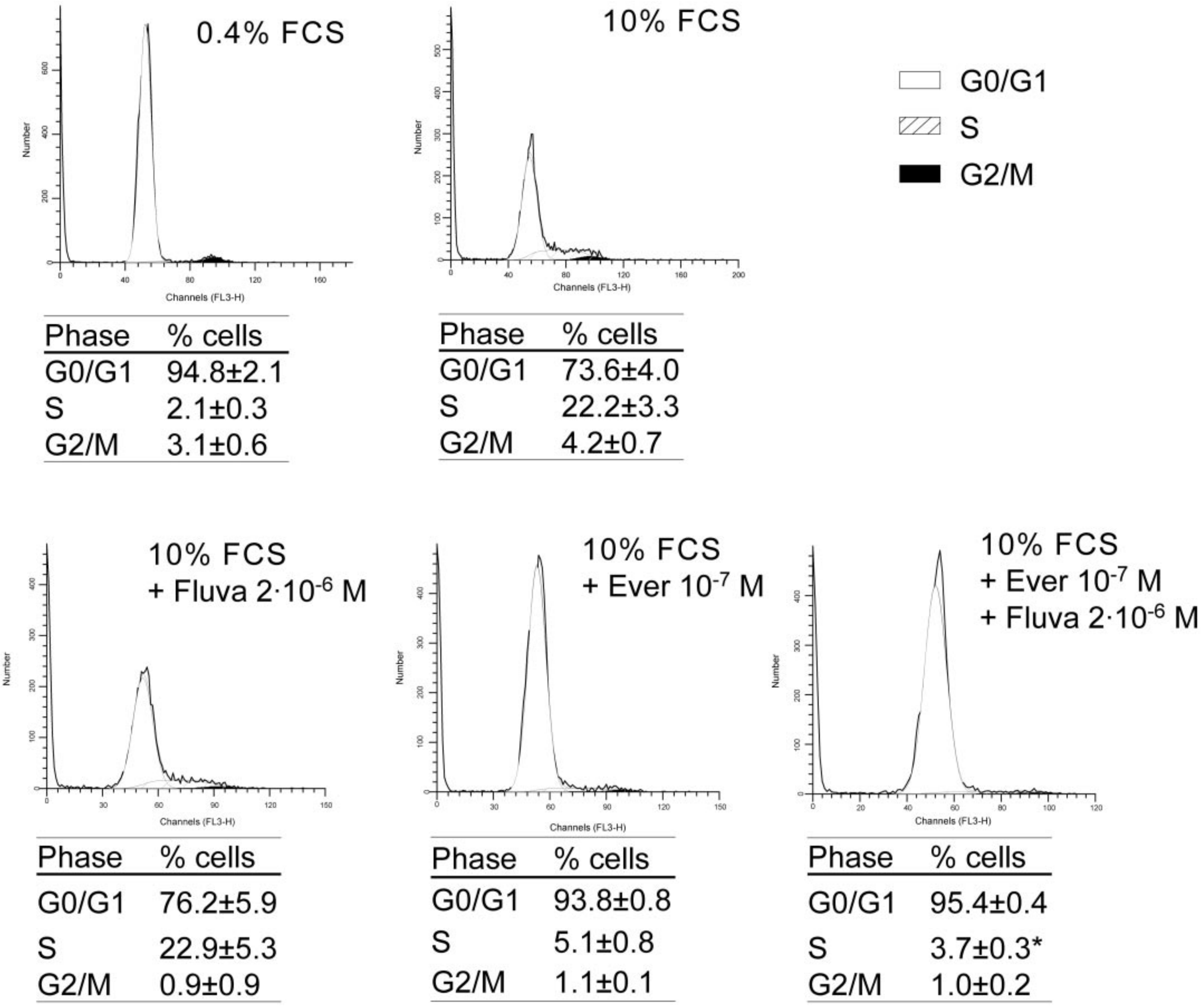
Everolimus (mol/L)	$[^3H]$-thymidine incorporation (% vs control)				R (expected/observed)
	Everolimus	Fluvastatin ($2\cdot10^{-6}$ mol/L)	Everolimus + Fluvastatin		
	alone	alone	expected	observed	
10^{-9}	60.7	85.8	52.0	32.1	1.62
10^{-8}	46.1		39.5	29.1	1.36
10^{-7}	36.7		31.5	24.1	1.31

Fig. 3. Effect of everolimus alone or in combination with fluvastatin on $[^3H]$thymidine incorporation. Cells were seeded at a density of 2×10^5 per 35-mm dish and incubated with DMEM supplemented with 10% FCS. Twenty-four hours later, the medium was changed with medium containing 0.4% FCS to stop cell growth, and the cultures were incubated for 5 days. At this time, the medium was replaced with medium containing 10% FCS, in the presence or absence of indicated concentrations of drugs. After 16 h, at 37°C, cells were labeled with $[^3H]$thymidine for 2 h, and radioactivity was evaluated. Each bar represents the mean ± S.D. of triplicate dishes. *, $p < 0.05$ and ***, $p < 0.001$, treatment versus control (Student's *t* test). The data are representative of three replicate experiments. Synergistic interaction between everolimus and fluvastatin is evidenced by the *R* value greater than unity (Kern et al., 1988).

served only in combination with fluvastatin. In contrast, everolimus alone, similar to rapamycin (Braun-Dullaeus et al., 2001), significantly affected cyclin E (−26.8%) and cyclin D1 expression (−36.2%), and the combination with fluvastatin enhanced the effect of everolimus on cyclin E (from −26.8 to −58.0%), but only slightly affect the expression of cyclin D1 (from −36.2 to −46.1%). As shown previously by others, up-regulation of the cyclin-dependent kinase inhibitor p21*Cip1* in response to 10% FCS was prevented by everolimus, although the addition of fluvastatin did not further increased this effect (Braun-Dullaeus et al., 2001). A significant change was also observed after the addition of fluvastatin to the action of everolimus on the levels of Rb hyperphosphorylation, which changed from −43.2 to −87.6%.

It was therefore conceivable to hypothesize that the reduction of cyclin E expression by combination of the two drugs may be responsible for the inhibition of the progression of the G_1 phase. The forced overexpression of cyclin E in rat SMC by retroviral infection conferred a partial but significant resistance to the antiproliferative action of the combination everolimus fluvastatin. This effect was observed on four different parameters related to cell proliferation: cell number after 3 days of exposure, [^{3}H]thymidine incorporation, cell cycle analysis of S phase, and Rb phosphorylation. These results suggest that the synergistic antiproliferative effect of the combination everolimus fluvastatin is partially mediated by the inhibition of the kinase activity of cdk2/cyclin E.

Everolimus and Fluvastatin Act on the Same Phase of the Cell Cycle by Inhibiting Different Intracellular Targets. Several studies have demonstrated that statins cause G_1 arrest by increasing cellular p27*kip1* levels and reducing cyclin E expression (Laufs et al., 1999; Rao et al., 1999; Fouty and Rodman, 2003). This effect has been ascribed to different prenylated proteins, including Ras and



0.4% FCS

Phase	% cells
G0/G1	94.8±2.1
S	2.1±0.3
G2/M	3.1±0.6

10% FCS

Phase	% cells
G0/G1	73.6±4.0
S	22.2±3.3
G2/M	4.2±0.7

10% FCS + Fluva $2 \cdot 10^{-6}$ M

Phase	% cells
G0/G1	76.2±5.9
S	22.9±5.3
G2/M	0.9±0.9

10% FCS + Ever 10^{-7} M

Phase	% cells
G0/G1	93.8±0.8
S	5.1±0.8
G2/M	1.1±0.1

10% FCS + Ever 10^{-7} M + Fluva $2 \cdot 10^{-6}$ M

Phase	% cells
G0/G1	95.4±0.4
S	3.7±0.3*
G2/M	1.0±0.2

Fig. 4. Effect of everolimus alone or in combination with fluvastatin on cell cycle of rat SMC. Experimental conditions are as in Fig. 3. Representative flow cytometry analysis of rat SMCs incubated with indicated concentrations of drugs are shown. Tables summarizing flow cytometry analysis of cell cycle performed in the presence of 0.4 and 10% FCS alone, or 10% FCS with reported concentrations of drugs are shown below each panel. The analysis was performed by using the ModFit LT software (Verity Software House). The data are representative of two replicate experiments. *, $p < 0.05$, everolimus versus everolimus + fluvastatin (Student's *t* test).

Rho GTP-binding proteins. Considerable evidence also indicates that mTORC1 inhibitors, such as rapamycin, similarly to statins, affect SMC proliferation by blocking in G_1 phase through a reduction of the expression of several cell cycle proteins, including cyclin D1 and cyclin E (Braun-Dullaeus et al., 2001), whereas its effect on $p27^{kip1}$ expression levels is still controversial (Gallo et al., 1999; Braun-Dullaeus et al., 2001).

In our study, we have shown that the combination of the two drugs significantly affects the expression of cyclin E and $p27^{kip1}$. The subliminal concentrations of fluvastatin used significantly affected the HMG-CoA reductase activity (Fig. 2) without altering cell proliferation and cell cycle molecule expression (Fig. 5). It is therefore tempting to speculate that fluvastatin, by reducing the intracellular synthesis of MVA and its isoprenoid derivatives, may alter turnover and function of Ras and/or Rho GTP-binding proteins, facilitating the antiproliferative effect of everolimus. This hypothesis is supported by the evidence that the coincubation with MVA, FOH or GGOH significantly prevented the additive effect of fluvastatin on the antiproliferative action of everolimus (Fig. 2C).

Evidence indicates that at least two farnesylated proteins and one geranylgeranylated protein are involved in the signaling of growth factors to mTORC1 complex: Ras, Rheb, and Cdc42 (Fang et al., 2003; Long et al., 2005; Ma et al., 2005). Ras/extracellular signal-regulated kinase pathway is responsible for the phosphorylation of tuberin event that negatively regulates tuberin function by blocking the interaction with hamartin, leading to the activation of farnesylated Rheb and mTORC1 complex (Shaw and Cantley, 2006). Alteration of the intracellular isoprenoid metabolism may therefore alter both Ras and Rheb, two positive regulators of mTORC1 complex. In agreement with this hypothesis, the specific farnesyl transferase inhibitor SCH66336 has been shown to efficiently inhibit Rheb prenylation and mTOR signaling associated with reduced levels of phosphorylated S6 (Basso et al., 2005). A second mode of mTORC1 regulation has been reported to occur via phosphatidic acid generated by phospholipase D. The activity of phospholipase D is known to be

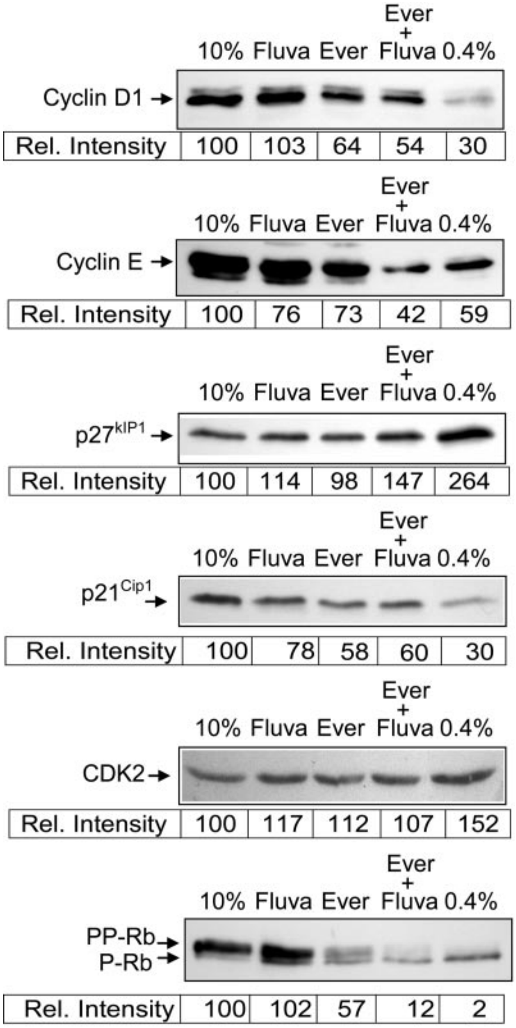


Fig. 5. Effect of everolimus alone or in combination with fluvastatin on cell cycle proteins. Experimental conditions are as in Fig. 2. Cell cycle protein expression levels were evaluated by Western blot analysis. The concentration of fluvastatin and everolimus was 2×10^{-6} and 10^{-7} M, respectively. Quantitative densitometric analysis was performed with GelDoc acquisition system and Quantity One software. The data are expressed as relative values, and they are representative of three replicate experiments.

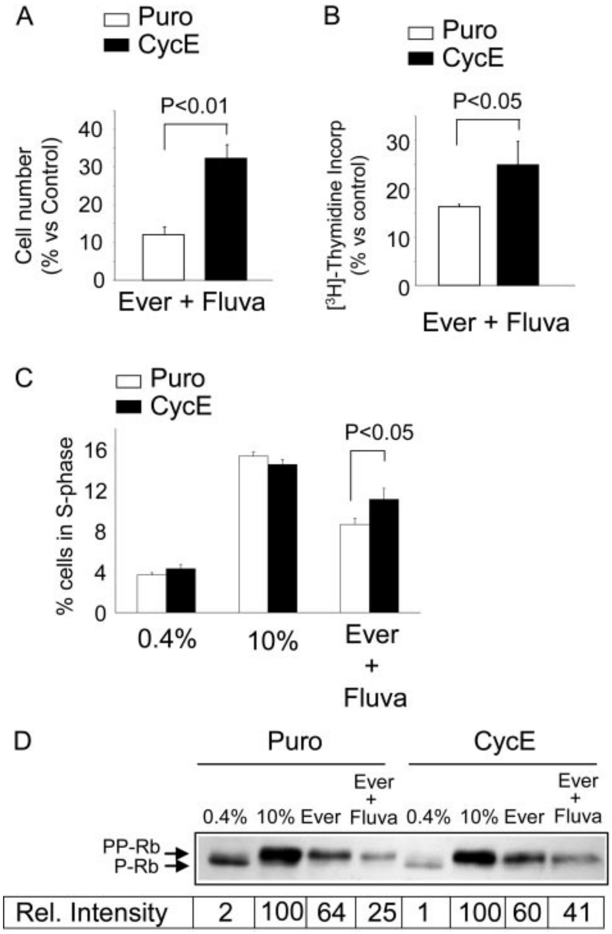


Fig. 6. Effect of cyclin E overexpression on the antiproliferative action of combination everolimus fluvastatin. A, antiproliferative action of combination everolimus (10^{-7} M) fluvastatin (5×10^{-7} M) was evaluated in rat SMCs transduced with pBM-IRES-PURO retrovirus encoding control vector (PURO) and cyclin E (CycE). B, the same cells described in A were used for determining the [^{3}H]thymidine incorporation after 16 h of incubation with combination everolimus (10^{-7} M) fluvastatin (2×10^{-6} M). The same experimental conditions described for B were used for cell cycle analysis (C) and the determination of Rb phosphorylation state by Western blot analysis (D). Each bar represents the mean ± S.D. of triplicates. The data are representative of two replicate experiments.

dependent on the small GTPase protein Cdc42 (Fang et al., 2003) and therefore potentially affected by the action of fluvastatin. It is noteworthy that the two modes of regulation of mTORC1 seem to interplay (Fang et al., 2003).

A second plausible explanation for the synergistic effect might be related to the interference of everolimus and fluvastatin on two distinct intracellular signaling pathways regulating the G_1 phase transition, mTORC1/p70S6 kinase and Rho/$p27^{kip1}$, respectively (Laufs et al., 1999). This possibility seems to be the most likely because everolimus by completely blocking the p70S6 kinase phosphorylation, a downstream effector of Ras, Rheb, and Cdc42, should not allow a further inhibition of this pathway by fluvastatin (Fig. 5).

Taken together, although the basic molecular mechanism that governs the synergistic effect of everolimus and fluvastatin is far from being understood, several indications point out a potential role of prenylated proteins. Future studies will be undertaken to identify which prenylated protein(s) is indeed involved in this process.

In conclusion, we provide evidence that everolimus and fluvastatin act synergistically to inhibit rat SMC proliferation in vitro, by altering the expression of cyclin E and $p27^{kip1}$, which affect Rb hyperphosphorylation leading to G_1 phase arrest. These results represent the basis for further experimental studies addressing the relevance of the synergistic properties of the combination everolimus and fluvastatin.

Acknowledgments

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Address correspondence to: Dr. Nicola Ferri, Department of Pharmacological Sciences, Via Balzaretti 9, 20133, Milan, Italy. E-mail: nicola.ferri@unimi.it

Correction to “Fluvastatin Synergistically Improves the Antiproliferative Effect of Everolimus on Rat Smooth Muscle Cells by Altering p27^{Kip1}/Cyclin E Expression”

In the above article [Ferri N, Granata A, Pirola C, Torti F, Pfister PJ, Dorent R, and Corsini A (2008) *Mol Pharmacol* **74:**144-153], the IC$_{50}$ units and values in Table 1 were incorrect because of an error during copyediting. The corrected table appears in its entirety below:

TABLE 1
Inhibitory effect of everolimus alone or in association with fluvastatin on cell proliferation

Assay	Inhibition (Conc.) for Fluvastatin	IC$_{50}$		Ratio	Maximal Effect (Conc.)	
		Everolimus	Everolimus + Fluvastatin		Everolimus	Everolimus + Fluvastatin
		nM				
Cell proliferation	−9.2 ± 8.4% (0.5 μM)	2.5	1.0	2.5	−54.9 ± 12.9% (0.5 μM)	−72.4 ± 8.3% (0.5 μM)
Thymidine incorporation	−14.2 ± 9.1% (2 μM)	6.5	0.19	34.2	−63.3 ± 4.6% (0.1 μM)	−75.9 ± 2.4% (0.1 μM)
Cell cycle (S phase)	3.2 ± 23.8% (2 μM)				−76.9 ± 3.8% (0.1 μM)	−83.4 ± 1.5% (0.1 μM)

The online version has been corrected in departure from the print version.

The printer regrets this error and apologizes for any confusion or inconvenience it may have caused.

Correction to “*Slc39a14* Gene Encodes ZIP14, A Metal/Bicarbonate Symporter: Similarities to the ZIP8 Transporter”

In the above article [Girijashanker K, He L, Soleimani M, Reed JM, Li H, Liu Z, Wang B, Dalton TP, and Nebert DW (2008) *Mol Pharmacol* **73:**1413-1423], some exponents were incorrectly reported as positive rather than negative. Under *Materials and Methods*, in the *Quantitative Polymerase Chain Reaction Analysis* section, the first sentence of the second paragraph should read “The range of calibration curves was linear from 10^{-3} to 10^{-7} μg of plasmid ($r = 0.999$ for ZIP14A, 0.998 for ZIPB, and 0.995 for ZIP8).”

The authors regret this error and apologize for any confusion or inconvenience it may have caused.