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Fluvastatin synergistically improves the antiproliferative effect of everolimus on rat smooth muscle cells by altering $p27^{kip1}$/cyclin E expression

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Running title: Combination everolimus fluvastatin and cell cycle progression.

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Abbreviations: Cyclin-dependent kinases (Cdks); fetal calf serum (FCS); 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA); retinoblastoma (Rb); internal ribosomal entry site (IRES); smooth muscle cell (SMC); eukaryotic translation initiation factor 4E (eIF4E); farnesol (FOH); geranylgeraniol (GGOH); eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1); mammalian target of rapamycin complex (mTORC); PBS, phosphate buffer saline; phospholipase D (PLD).

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Abstract

Multiple intracellular signaling pathways stimulate quiescent smooth muscle cells (SMCs) to exit from G0 and reenter the cell cycle. Thus, a combination of two drugs with different mechanism 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, an mTOR 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 ($5x10^{-7}$ M), lowering the IC_{50} value from $2.5x10^{-9}$ M to $1.0x10^{-9}$ 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 G1 phase. In particular, the drug combination significantly upregulated $p27^{Kip1}$ levels by 47.0%, suppressed cyclin E by 43.0%, and reduced retinoblastoma (Rb) hyperphosphorylation by 79.0%, compared to 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 affect Rb phosphorylation and leading to G1 phase arrest.

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Introduction

Smooth muscle cell (SMC) proliferation in the arterial wall is the major determinants of restenosis after balloon angioplasty and stent coronary implantation (Hansson, 2005; Ross, 1999). 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 has been re-evaluated (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 (Boden et al., 2007; Mody et al., 2001). In particular, a combination of two different pharmacological inhibitors capable to antagonize different intracellular signaling pathways involved in cell cycle reentry, may lead to a better control of SMCs proliferation.

The 40-O-(2-hydroxyethyl)-derivative of rapamycin, everolimus, is a proliferation signal inhibitor that affects growth factor–induced proliferation of haematopoietic and non-haematopoietic cells via cell cycle arrest at the late G1 phase (Brown et al., 1995; Decker et al., 2003; Hafizi et al., 2004; Price et al., 1992). The antiproliferative action of everolimus is elicited through binding to the mammalian target of rapamycin complex 1 (mTORC1) complex composed of mTOR, a common regulatory subunit called LST8, and the raptor subunit that specifies the downstream substrates (Sarbassov et al., 2004; Schuler et al., 1997; 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, the p70 S6 protein kinase (p70S6) and the eukaryotic translation initiation factor 4E (eIF4E)-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 blocking

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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 immunosuppressant, prevention of renal and heart transplant rejection, and retardation of cardiac allograft vasculopathy (Nashan, 2002; Schuler et al., 1997). 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 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also called statins. We have previously shown that fluvastatin interferes with SMC proliferation *in vitro* at therapeutic concentrations ($0.1\text{-}1x10^{-6}$ 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 appears 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 (Bellosta et al., 2000; Corsini et al., 1993; Raiteri et al., 1997). Several prenylated proteins belonging to different intracellular signaling pathways have been documented to be indispensable for cell proliferation, including

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the small GTP-binding protein Ras, and Ras-like proteins, such as Rho, Rac and Rap (Brown et al., 2006; Corsini et al., 1999).

Interestingly, the combination of fluvastatin and everolimus has previously shown 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, although the basic molecular mechanisms 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 and fluvastatin on SMC proliferation and the underling molecular mechanisms.

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Material and methods

Reagents and antibodies

DMEM, trypsin ethylendiaminetetraacetate, penicillin (10,000 U ml^{-1}), streptomycin (10 mg ml^{-1}), tricine buffer (1 M, pH 7.4) and nonessential amino acid solution (100X), fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA, USA). Disposable culture flasks and petri dishes were from Corning Glassworks (Oneonta, New York), and filters were from Millipore (Billerica, MA ,USA). [6-^{3}H]-Thymidine, sodium salt (2 Ci mM^{-1}) was from Amersham (Cologno Monzese, Milan, Italy), and molecular weight protein standards from BIO-RAD Laboratories (Hercules, CA, USA). 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 (Hercules, CA, USA). Cytox-Dye was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). 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. For western blot analysis the following antibodies have been utilized: anti-Cyclin D1, anti-cyclin E, anti-cdk2, anti-p70 S6 kinase, and anti-phospho-p70S6 kinase Thr-412 (Upstate, Lake Placid, NY, USA), anti-$p27^{Kip1}$ (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), anti-Rb protein (Chemicon International Temecula, CA, USA), anti 4E-BP1 (Cell signaling Technology), anti-$p21^{Cip1}$ (Abcam plc, Cambridge, UK), anti-mouse and anti-rabbit peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Lab, West Grove, PA, USA).

Cell proliferation and DNA synthesis

SMCs were cultured from the intimal-medial layers of aorta of male Sprague-Dawley rats as previously described (Corsini et al., 1995). Cells were seeded at a density of $1 \cdot 10^{5}$ SMC/Petri

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dish (35 mm), and incubated with DMEM supplemented with 10% FCS. 24 h later, the medium was changed to 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 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 (Coulter Instruments) 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 to cell number found in each triplicate after 72 h of cell growth.

For DNA synthesis, synchronization of SMCs to the G0/G1 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 a 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 utilized 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 to 40 μg) were assayed in a buffer containing 0.25 mol/L K_2HPO_4 (pH 7.4), 100 mmol/L glucose-6-phosphate, 15 mmol/L NADP, 50 mmol/L dithiothreitol, and 110 μmol/L 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 minutes before the addition of HMG-CoA and then incubated for 120 minutes at 37°C with

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moderate shaking. The reaction was stopped by the addition of 20 µL of 5 mol/L 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 minutes to allow lactonization of the mevalonate. The mixture was extracted twice with 10 mL (20 mL total) of diethyl ether. The upper 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 utilized were the same than those utilized for DNA synthesis assay. Flow cytometry was utilized to analyze cell cycle distribution. Cells were trypsinized and centrifuged for 5 min at 1.000 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 $MgCl_2$ 0.1% NP-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 FACS scan flow cytometer (Becton Dickinson) and analyzed with ModFit LT software (Verity Software House, Topsham, ME, USA). The number of cells in G0/G1, S and G2/M phases was expressed as percentages of total events (10.000 cells) (Ferri et al., 2003).

Western blot analysis

Cells were washed twice with PBS and lysed by incubation with a solution of 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, containing a protease and phosphatase inhibitor

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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 BCA protein assay (Pierce). Lysates were separated by SDS-PAGE under reducing conditions, transferred to Immobilon PVDF (Millipore) and subsequently immunoblotted with primary antibody following appropriate secondary antibody, prior to visualization by enhanced chemiluminescence (ECL, Amersham Biosciences). Quantitative densitometric analyses were performed using Gel Doc acquisition system and Quantity One software (BIO-RAD).

Generation of cyclin E expression construct and retroviral infection

Full-length rat cyclin E (accession #: D14015) was generated by PCR using the following primers: 5' ATGAAAGAAGAAGGTGGTTCCG 3' and 5' TCATTCTGTCTCCTGCTCACTGC 3'. The sequence of the PCR-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 G.P. Nolan (Stanford University, Stanford, CA, USA). Retroviral infections of human SMC were performed as previously described (Garton et al., 2002).

Analysis of drug synergism

According to the method of Kern et *al* the expected value of cell number (CN_{exp}, defined as the product of the percentage *vs* control of cell number observed after incubation with drug A alone and the percentage of cell number observed for drug B alone divided by one hundred) and the actual cell number observed (CN_{obs}) for the combination of A and B were used to construct a synergistic ratio as follows:

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$R = CN_{exp} / CN_{obs}$

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 ± SD. 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 non linear regression curve (SigmaPlot software).

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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 $5x10^{-10}$ to $5x10^{-7}$ 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 $2.5x10^{-9}$ M. Although everolimus very potently reduced cell proliferation, it did not allow a complete inhibition with 54.9±12.9% inhibitory effect at $5x10^{-7}$ M (Fig 1A). A *plateau* of about 55% inhibition was reached at concentrations of $5x10^{-9}$ M and above (Fig 1A).

The inhibitory effect of everolimus on SMC proliferation was then evaluated in combination with subliminal fluvastatin concentration ($5x10^{-7}$ M). Fluvastatin alone resulted in a non-significant 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} equal to $1.0x10^{-9}$ 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 inhibitory effect of 72.4±8.3% at $5x10^{-7}$ M everolimus concentration (Fig 1A and Table1).

To determine whether the antiproliferative action of fluvastatin in combination with everolimus was additive or synergistic, data were analyzed according to Kern et *al* (Kern et al., 1988). The combination of the two drugs produced an additive effect until $5x10^{-9}$ M and a synergistic effect with higher concentrations with R values of 1.35 and 1.47 at 10^{-8} and $5x10^{-7}$ M, respectively (Fig 1C).

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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, $5x10^{-7}$M fluvastatin upregulated 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, 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 ($5x10^{-7}$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.

Since 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, 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 co-incubation with MVA, FOH or GGOH, abolished the synergistic antiproliferative

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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 G1 phase

Everolimus has been reported to induce a cell cycle arrest at the G1 phase by blocking mTOR activity (Azzola et al., 2004; Schuler et al., 1997), 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 $6.47x10^{-9}$ M. The combination of everolimus with $2x10^{-6}$ 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 $1.9x10^{-10}$ M (Fig 3A and Table1).

We next studied the effect of everolimus in association with fluvastatin, on the progression of the cell cycle from G1 to S phase by flow cytometry analysis. Incubation of SMCs with 0.4% FCS led to accumulation of cells at G0/G1 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). Importantly, we did not observe any significant increase in the percentage of cells at sub-G0/G1 phase, demonstrating a specific antiproliferative activity of everolimus without any induction of apoptosis (Fig 4). Although

everolimus almost completely inhibited the progression from G1 phase to S phase, the combination with $2x10^{-6}$ 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 G1 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 G1 phase, we carried out a series of experiments aiming at evaluating the expression levels of cyclins expressed in the G1/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 to 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 (Fig 3 and 4), did not significantly change both cyclin D1 and E expression levels. However, 10^{-7} M everolimus reduced by 36.2% and 26.8% cyclin D1 and 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 $p21^{Cip1}$. While $p27^{Kip1}$ 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 to 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).

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, 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%), condition similar to that observed in quiescent cells (0.4% FCS), indicating a cell cycle arrest in G1.

Altogether, the present results indicate that everolimus affected SMC proliferation by interfering with the progression of the G1 phase reducing the expression of both cyclin D1 and 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 human SMCs as compared to cells transduced with PURO control vector (data no shown). These established cell lines were then utilized for determining the antiproliferative action of the combination fluvastatin everolimus. After three days, the combination of the two drugs (fluvastatin $5 \cdot 10^{-7}$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

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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 $2 \cdot 10^{-6}$ M and everolimus 10^{-7}M) (Fig 6B, C and D). Indeed, [^{3}H]-thymidine incorporation in control and cyclin E-overexpressing cells were equal to 16.3±0.4% and 24.9±4.8% vs 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 were replicating the DNA (8.64±0.6 %) compared to 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 to 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 downregulation is required for a full exploitation of the inhibition of cell growth by these two drugs.

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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 three days of exposure to the drugs and by [^{3}H]-thymidine incorporation assay after 16 h of incubation (Table 1). Moreover, by a biological and pharmacological approaches and genetic modification of rat SMCs we demonstrated that the synergistic effect of this drug combination converge on the regulation of cyclinE/p27^{kip1} complex leading to a block in G1 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 [^{3}H]-thymidine incorporation, respectively (Table 1). In terms of efficacy, everolimus led to a growth inhibition up to 54.9±12.9%, 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 chemotherapic 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 then 5x10^{-9} M

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The synergistic antiproliferative effect of everolimus fluvastatin is elicited in G1 phase of the cell cycle by affecting p27^{kip1}/cyclin E expression

Since 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 G1/S phase, we studied the expression levels of G1 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 and E and A (Adams, 2001). In particular the expression of D type cyclins appears 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 (Aleem et al., 2005; Ekholm and Reed, 2000), and are believed to complete the phosphorylation of pRb, initiated by the action of cyclin D-cdk complexes (Fu et al., 2004; Stacey, 2003). More recently the generation of cyclin E knock out mice allowed to demonstrate their essential function in cell cycle reentry (Geng et al., 2003). In our study, the antiproliferative effect of the tested drugs was assessed in SMCs previously synchronized in G0 phase of the cell cycle, and then stimulated by the addition of 10% FCS. Under these experimental conditions the upregulation 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 upregulation (+47.0%) was observed 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 -

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58.0%), but only slightly affect the expression of cyclin D1 (from -36.2% to -46.1%). As previously shown by others, upregulation 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 G1 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/cyclinE.

Everolimus and fluvastatin act on the same phase of the cell cycle by inhibiting different intracellular targets

A number of studies have demonstrated that statins cause G1 arrest by increasing cellular $p27^{kip1}$ levels and reducing cyclin E expression (Fouty and Rodman, 2003; Laufs et al., 1999; Rao et al., 1999). This effect has been ascribed to different prenylated proteins including Ras and Rho GTP-binding proteins. Several evidences also indicate that mTORC1 inhibitors, such as rapamycin, similarly to statins, affect SMC proliferation by blocking in G1 phase through a reduction of the expression of several cell cycle proteins, including cyclin D1 and cyclin E

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(Braun-Dullaeus et al., 2001), while its effect on $p27^{kip1}$ expression levels is still controversial (Braun-Dullaeus et al., 2001; Gallo et al., 1999).

In our study, we have shown that the combination of the two drugs significantly affect the expression of cyclin E and $p27^{kip1}$. The subliminal concentrations of fluvastatin utilized significantly affected the HMG-CoA reductase activity (Figure 2) without altering cell proliferation and cell cycle molecules expression (Figure 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 co-incubation with MVA, FOH or GGOH significantly prevented the additive effect of fluvastatin on the antiproliferative action of everolimus (Fig 2C).

Existing evidence indicate 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/ERK pathway is responsible for the phosphorylation of tuberin (TSC2) event that negatively regulates TSC2 function by blocking the interaction with TSC1 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 the phospholipase D (PLD). The activity of PLD is known to be dependent by the small GTPase protein, Cdc42 (Fang et al., 2003), and therefore potentially affected by the

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action of fluvastatin. Importantly, the two mode of regulations of mTORC1 seem to act in an interplayed manner (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 G1 phase transition, mTORC1/p70S6 kinase and Rho/$p27^{kip1}$ respectively (Laufs et al., 1999). This possibility appears to be the most likely since 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 G1 phase arrest. These results represent the basis for further experimental studies addressing the relevance of the synergistic properties of the combination everolimus fluvastatin.

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Footnote

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Legends for figures

Figure 1. *Effect of everolimus alone or in combination with fluvastatin on rat SMC proliferation.*

(A) Cells were seeded at a density of 1 x 10^5/35 mm ∅ dish and incubated with DMEM supplemented with 10% FCS; 24 h later, the medium was changed with one containing 0.4% FCS to stop cell growth and the cultures were incubated for 72 h. At this time, the medium was replaced with one 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 ± SD of six different experiments. *p<0.05; everolimus *vs* everolimus + fluvastatin (Student's T-test). (B) The experimental conditions are the same as panel A. Each bar represents the mean ± SD of three different experiments; *p<0.05; **p<0.001; fluvastatin *vs* control (Student's T-test). (C) The synergistic effect of the combination everolimus with fluvastatin is demonstrated by the *R* value greater then unity. The synergistic ratio represents the ratio of expected inhibitory effect on cell proliferation and the observed inhibition (Kern et al., 1988).

Figure 2. *Effect of everolimus alone or in combination with fluvastatin on HMG-CoA reductase activity, 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 ($5 \cdot 10^{-7}$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 in "Material and Methods". Each bar represents the mean ± SD of triplicate samples. (B) Under the same experimental conditions described for panel A, p70S6 kinase

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(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 (Upstate, Lake Placid, NY, USA). 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 ($5 \cdot 10^{-7}$M), everolimus (10^{-8}M and 10^{-9}M) and their combination in the presence or absence of MVA (10^{-4}M), FOH (10^{-5}M) or GGOH ($5 \cdot 10^{-6}$). Each bar represents the mean ± SD of two different experiments. Statistical analysis was performed by Student's T-test. NS: not significant.

Figure 3. *Effect of everolimus alone or in combination with fluvastatin on [^{3}H]-thymidine incorporation*

(A) Cells were seeded at a density of 2 x 10^5/35 mm ∅ dish and incubated with DMEM supplemented with 10% FCS; 24 h later, the medium was changed with one containing 0.4% FCS to stop cell growth and the cultures were incubated for 5 days. At this time, the medium was replaced with one containing 10% FCS, in the presence or absence of indicated concentrations of drugs. After 16 h, at 37°C, cells were labelled with [^{3}H]-thymidine for 2 h and radioactivity was evaluated. Each bar represents the mean ± S.D. of triplicate dishes. *p<0.05; ***p<0.001 treatment *vs* control (Student's T-test). The data are representative of three replicate experiments. (B) The synergistic interaction between everolimus and fluvastatin is evidenced by the *R* value greater then unity (Kern et al., 1988).

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Figure 4. *Effect of everolimus alone or in combination with fluvastatin on cell cycle of rat SMC* Experimental conditions are as in Figure 3. Representative flowcytometry 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%, 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, Topsham, ME, USA). The data are representative of two replicate experiments. *p<0.05; everolimus *vs* everolimus + fluvastatin (Student's T-test).

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

Figure 6. *Effect of cyclin E overexpression on the antiproliferative action of combination everolimus fluvastatin.* (A) The antiproliferative action of combination everolimus (10^{-7}M) fluvastatin ($5x10^{-7}$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 for panel A were utilized for determining the [^{3}H]-thymidine incorporation after 16 h of incubation with combination everolimus (10^{-7}M) fluvastatin ($2 \cdot 10^{-6}$M). The same experimental conditions described for panel B were utilized for cell cycle analysis (C) and the determination of Rb

phosphorylation state by western blot analysis (D). Each bar represents the mean ± SD of triplicates. The data are representative of two replicate experiments.

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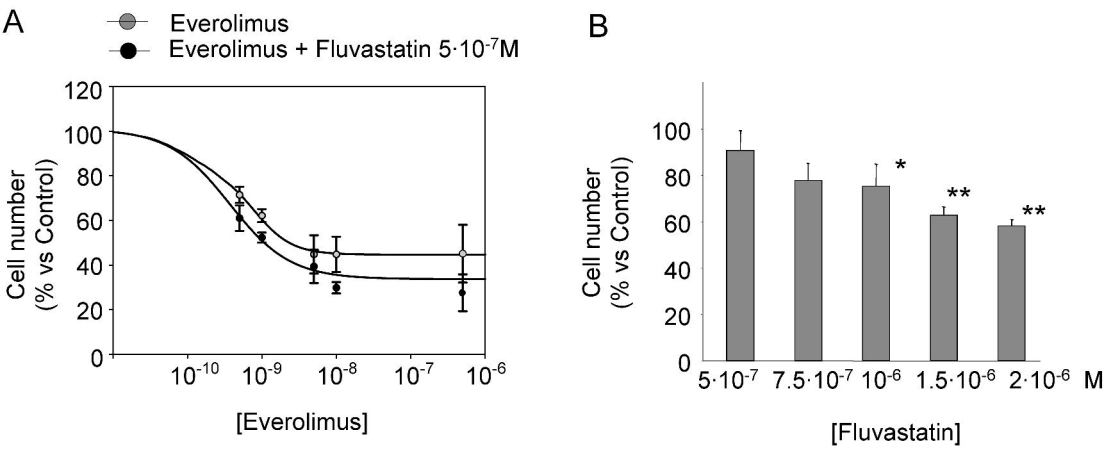
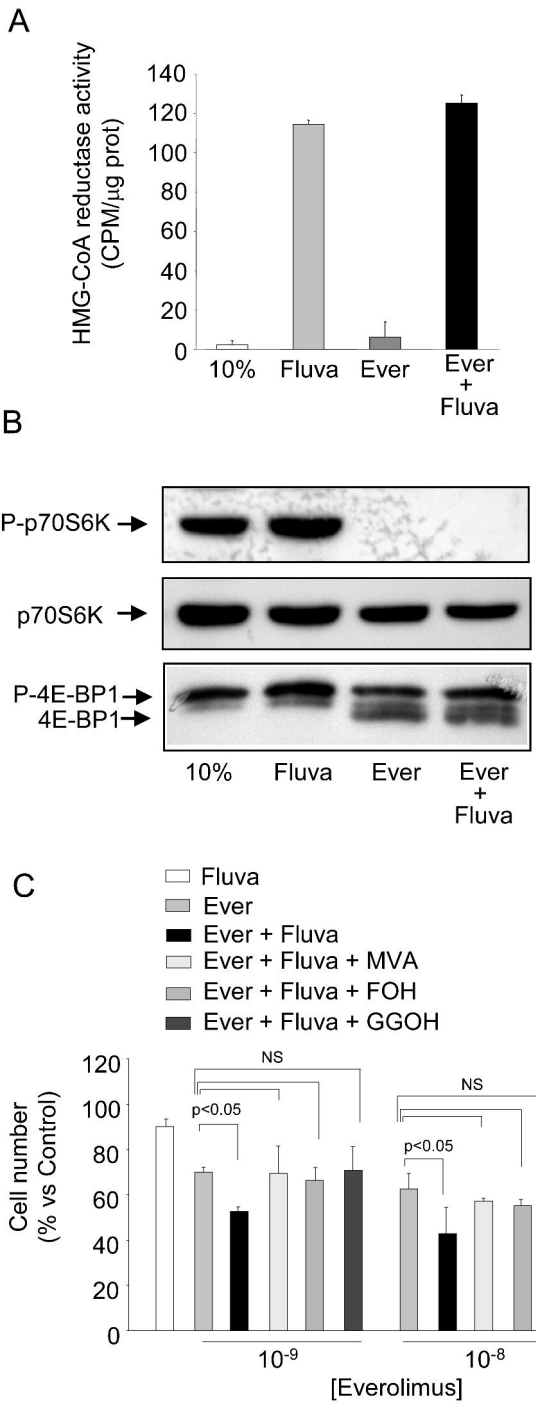
Table 1. Inhibitory effect of Everolimus alone or in association with fluvastatin on cell proliferation.

Assay	**Fluvastatin**	**Everolimus**	**Everolimus + Fluvastatin**		**Everolimus**	**Everolimus + Fluvastatin**
	% inhibition (Concentration)	IC_{50}		Ratio	Maximum effect (Concentration)	
Cell proliferation	-9.2±8.4% ($5x10^{-7}$ M)	$2.5x10^{-9}$ M	$1.0x10^{-9}$ M	2.5	-54.9±12.9% ($5x10^{-7}$M)	-72.4±8.3% ($5x10^{-7}$M)
Thymidine Incorporation	-14.2±9.1% ($2x10^{-6}$M)	$6.5x10^{-9}$M	$1.9x10^{-10}$ M	32.7	-63.3±4.6% ($1x10^{-7}$M)	-75.9±2.4% ($1x10^{-7}$M)
Cell cycle (S phase)	+3.2±23.8% ($2x10^{-6}$M)				-76.9±3.8% ($1x10^{-7}$M)	-83.4±1.5% ($1x10^{-7}$M)

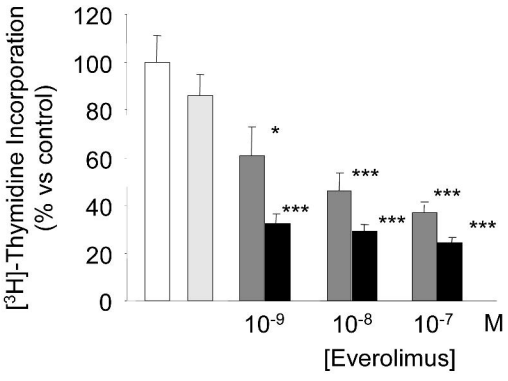


Everolimus (mol/L)	Cell number (% vs control)				R (expected/observed)
	Everolimus alone	Fluvastatin ($5\cdot10^{-7}$ mol/L) alone	Everolimus + Fluvastatin expected	Everolimus + Fluvastatin observed	
$5\cdot10^{-10}$	71.5	89.8	64.1	61.1	1.05
10^{-9}	62.2		55.8	52.3	1.07
$5\cdot10^{-9}$	44.8		40.2	39.4	1.02
10^{-8}	44.8		40.2	29.8	1.35
$5\cdot10^{-7}$	45.1		40.5	27.6	1.47

FIGURE 1



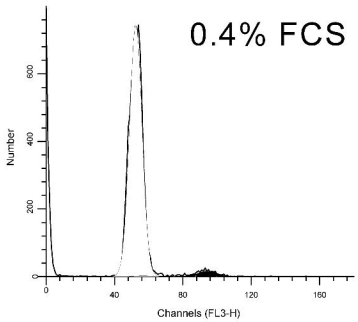
FERRI et al, FIGURE 2



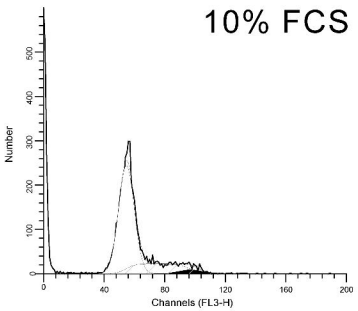
10% FCS
10% FCS + Fluvastatin $2 \cdot 10^{-6}$ M
10% FCS + Everolimus
10% FCS + Everolimus + Fluvastatin $2 \cdot 10^{-6}$ M

Everolimus (mol/L)	[3H]-thymidine incorporation (% vs control)				R (expected/observed)
	Everolimus alone	Fluvastatin ($2 \cdot 10^{-6}$ mol/L) alone	Everolimus + Fluvastatin expected	Everolimus + Fluvastatin 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

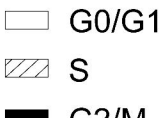
FIGURE 3

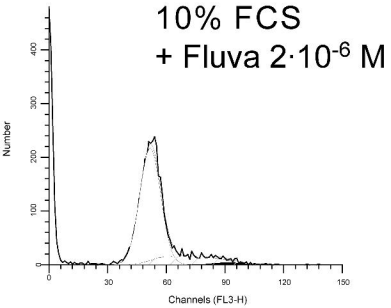


Phase	% cells
G0/G1	94.8±2.1
S	2.1±0.3
G2/M	3.1±0.6

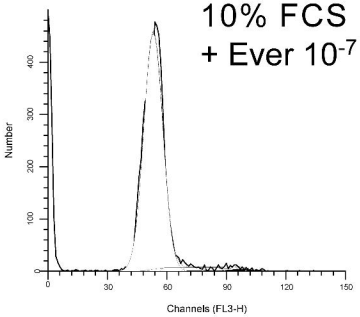


Phase	% cells
G0/G1	73.6±4.0
S	22.2±3.3
G2/M	4.2±0.7

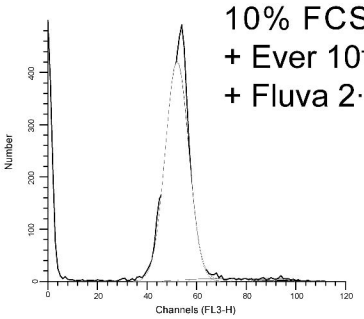




Phase	% cells
G0/G1	76.2±5.9
S	22.9±5.3
G2/M	0.9±0.9



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



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

FIGURE 4

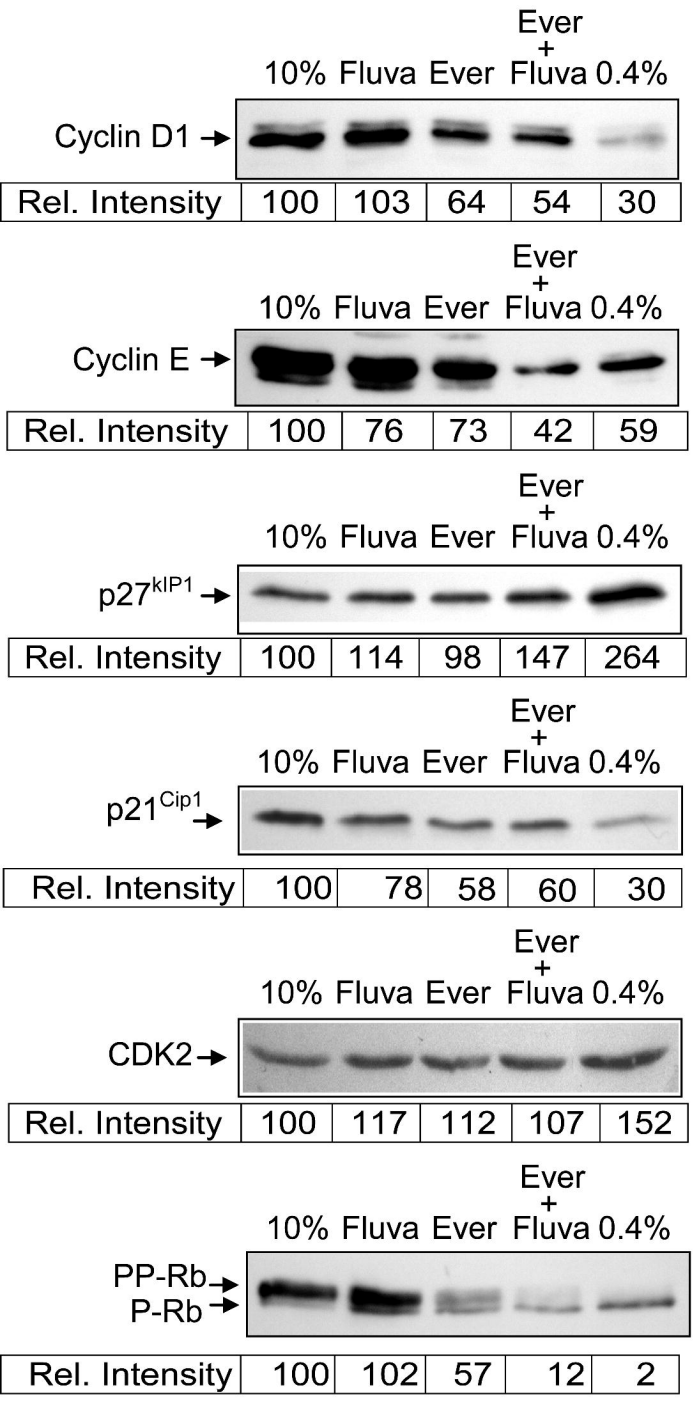

10% Fluva
Ever
Ever + Fluva 0.4%
Cyclin D1
Rel. Intensity 100 103 64 54 30
Cyclin E
Rel. Intensity 100 76 73 42 59
p27kIP1
Rel. Intensity 100 114 98 147 264
p21Cip1
Rel. Intensity 100 78 58 60 30
CDK2
Rel. Intensity 100 117 112 107 152
PP-Rb
P-Rb
Rel. Intensity 100 102 57 12 2

FIGURE 5

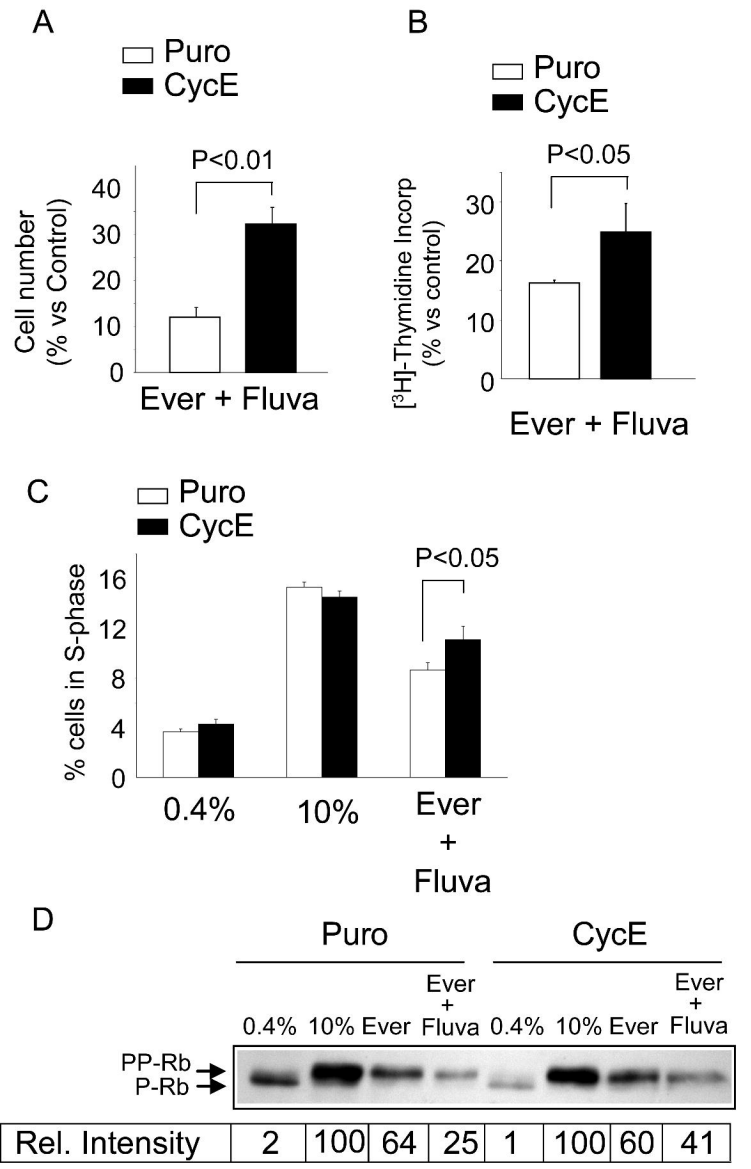

A
Puro
CycE
P<0.01
Cell number
(% vs Control)
40
30
20
10
0
Ever + Fluva
B
Puro
CycE
P<0.05
[3H]-Thymidine Incorp
(% vs control)
30
20
10
0
Ever + Fluva
C
Puro
CycE
P<0.05
% cells in S-phase
16
12
8
4
0
0.4%
10%
Ever
+
Fluva
D
Puro
CycE
Ever
+
Fluva
0.4% 10% Ever
Ever
+
Fluva
0.4% 10% Ever
PP-Rb
P-Rb
Rel. Intensity
2
100
64
25
1
100
60
41

FIGURE 6