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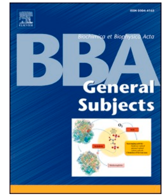

Cladosporols A and B, two natural peroxisome proliferator-activated receptor gamma (PPARγ) agonists, inhibit adipogenesis in 3T3-L1 preadipocytes and cause a conditioned-culture-medium-dependent arrest of HT-29 cell proliferation

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ABSTRACT

Background: Obesity and type 2 diabetes mellitus, which are widespread throughout the world, require therapeutic interventions targeted to solve clinical problems (insulin resistance, hyperglycaemia, dyslipidaemia and steatosis). Several natural compounds are now part of the therapeutic repertoire developed to better manage these pathological conditions. Cladosporols, secondary metabolites from the fungus *Cladosporium tenuissimum*, have been characterised for their ability to control cell proliferation in human colon cancer cell lines through peroxisome proliferator-activated receptor gamma (PPARγ)-mediated modulation of gene expression. Here, we report data concerning the ability of cladosporols to regulate the differentiation of murine 3T3-L1 preadipocytes.
Methods: Cell counting and MTT assay were used for analysing cell proliferation. RT-PCR and Western blotting assays were performed to evaluate differentiation marker expression. Cell migration was analysed by wound-healing assay.
Results: We showed that cladosporol A and B inhibited the storage of lipids in 3T3-L1 mature adipocytes, while their administration did not affect the proliferative ability of preadipocytes. Moreover, both cladosporols downregulated mRNA and protein levels of early (C/EBPα and PPARγ) and late (aP2, LPL, FASN, GLUT-4, adiponectin and leptin) differentiation markers of adipogenesis. Finally, we found that proliferation and migration of HT-29 colorectal cancer cells were inhibited by conditioned medium from cladosporol-treated 3T3-L1 cells compared with the preadipocyte conditioned medium.
Conclusions: To our knowledge, this is the first report describing that cladosporols inhibit *in vitro* adipogenesis and through this inhibition may interfere with HT-29 cancer cell growth and migration.
General significance: Cladosporols are promising tools to inhibit concomitantly adipogenesis and control colon cancer initiation and progression.

1. Introduction

Obesity is a risk factor for several diseases such as type 2 diabetes, metabolic syndrome, hypertension, hyperlipidaemia, atherosclerosis and cancer. Hyperplasia (an increase in adipocyte numbers) and hypertrophy (an increase in adipocyte mass) both lead to obesity [1]. Genetics, metabolism, nutritional status, exercise and, in general, habits of life may differentially contribute to the development of obesity.

Abbreviations: BAT, brown adipose tissue; CRC, colorectal cancer; DMEM, Dulbecco's Modified Eagle Medium; FACS, fluorescence-activated cell sorting; FBS, foetal bovine serum; FCS, foetal calf serum; IBMX, 3-isobutyl-1-methylxanthine; MDI, differentiation medium; mRNA, messenger RNA; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PPARγ, peroxisome proliferator-activated receptor gamma; PPRE, PPAR response element; RGZ, rosiglitazone; RXR, retinoid X receptor; TZD, thiazolidinedione;; WAT, white adipose tissue.

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Endogenous lipogenesis and storage of external lipids in mature adipocytes are relevant steps in adipogenesis [2]. For these reasons, reduction of adipose depots through inhibition of lipogenesis and the simultaneous promotion of lipolysis have been identified as crucial therapeutic interventions for obesity treatment [3,4]. Investigations about molecules that interfere with preadipocyte differentiation to mature adipocytes have received considerable attention from clinical and molecular researchers. Indeed, an increasing number of papers have demonstrated that several natural compounds may inhibit adipogenesis and lipogenesis, promote lipolysis and also induce apoptosis of adipocytes [5].

Peroxisome proliferator-activated receptor gamma (PPARγ or NR1C3), together with its isoforms PPARα (NR1C1) and PPARβ/δ (NP1X2), are type II nuclear receptors that are usually inactive in the nucleus until they bind to a ligand [6,7]. Ligand binding causes a conformational change in the receptor structure that, in turn, provokes the displacement of corepressors and the recruitment of coactivators. Ligand-activated PPARγ forms a heterodimer with retinoid X receptor (RXR); this heterodimer binds to the PPAR response element (PPRE) and stimulates the transcription of several gene targets that, consequently, modulate the metabolism of carbohydrates and lipids, cell proliferation and differentiation, angiogenesis and inflammation [8]. In humans, four distictive messenger RNAs (mRNAs) are transcribed from the unique PPARγ gene; they result from different promoter usage and alternative splicing. PPARγ1, PPARγ3 and PPARγ4 mRNAs generate the same protein product, namely PPARγ1 (477 amino acids), while PPARγ2 mRNA produces the PPARγ2 isoform, which contains 30 additional amino acids at the N-terminus (507 amino acids) [9]. The PPARγ1 isoform is expressed in many tissues including white and brown adipose tissue (WAT and BAT, respectively), the heart, the kidneys, immune cells such as macrophages, skeletal muscle, the pancreas and the colon. Conversely, the expression of PPARγ2 is restricted to adipose tissue but may be induced in other tissues by a high-fat diet [10,11]. PPARγ is considered a master regulator gene of adipocyte differentiation and plays a key role in adipogenesis. Analysis of the PPARγ-null mouse, which lacks adipose tissue, definitively established this crucial function [12]. PPARγ has also been found in an adipocyte progenitor population within the WAT perivascular niche; this finding suggests that it may play a pivotal role in adipocyte self-renewal [13]. Finally, PPARγ is necessary to maintain the mature adipocyte phenotype because selective PPARγ ablation in this cell type only allows survival for a few days, followed by death [14].

Besides its role in adipocyte differentiation and lipid metabolism, PPARγ controls the gene networks involved in glucose homeostasis. Moreover, it modulates the synthesis and secretion of various adipokines (adiponectin, resistin, leptin and tumour necrosis factor [TNF-α]), which control insulin sensitivity [15]. PPARγ target genes may exert their functions in different tissues, and thus they can positively influence insulin sensitivity through a wide range of mechanisms. For example, TNF-α acts on inflammation, adiponectin on glucose utilisation and insulin on nutrient uptake. Administration of PPARγ ligands may be considered a powerful therapeutic intervention capable of targeting multiple overlapping pathways and directed to solve different dysfunctions in a multifactorial disease such as diabetes and obesity. Natural and synthetic ligands have been found to bind PPARγ; among them are the thiazolinediones (TZDs), which at one time were the most used sensitisers of insulin signaling in the treatment of type 2 diabetes mellitus [16,17]. Unfortunately, their use was associated with side effects and adverse events (hepatic injury, cardiovascular events, weight gain, fluid retention, bone fractures, oedema and cancer); hence, they have been removed from the market or their use is restricted [18–21].

We recently demonstrated that cladosporol A, a secondary metabolite from the fungus *Cladosporium tenuissimum*, exhibits antiproliferative properties in a variety of human colon cancer cell lines through PPARγ-mediated modulation of gene expression of several cell cycle gatekeepers ($p21^{waf1/cip1}$, cyclin D1, cyclin E, CDK2 and CDK4) [22,23]. These findings are consistent with results from the literature that PPARγ plays a protective role in *in vitro* and *in vivo* colorectal cancer (CRC) models [24,25]. We also investigated cladosporol B, an oxidised form of cladosporol A, and demonstrated that it is more efficient in inhibiting cell proliferation and inducing apoptosis in HT-29 cells, a CRC cell line. Cladosporol B acts as a PPARγ partial agonist, with lower affinity and reduced transactivation potential after transient transfection compared with the full agonists cladosporol A and rosiglitazone (RGZ, a TZD). Docking experiments provided the structural basis for full and partial PPARγ agonism of cladosporol A and B, respectively, confirming that the binding of cladosporol B is associated with a lower transactivation potential, higher antiproliferative activity and more pronounced proapoptotic activity compared with the two full agonists cladosporol A and RGZ [26].

We further investigated the properties of cladosporol A and cladosporol B in a different cellular context, namely the 3T3-L1 cell line, an *in vitro* model of preadipocytes. In this work, we demonstrated that cladosporol A and cladosporol B inhibited adipogenesis in 3T3-L1 preadipocytes that are committed to differentiate to mature adipocytes through an induction cocktail (3-isobutyl-1-methylxanthine, dexamethasone, insulin [MDI] and foetal bovine serum [FBS]) for 10 days. Using real-time polymerase chain reaction (PCR) and western blotting, we also verified that the mRNA and protein levels of early and late adipogenic markers were significantly reduced by treatment with cladosporol A or cladosporol B. Finally, we demonstrated that culture medium from 3T3-L1 cells treated with cladosporol A or cladosporol B could inhibit proliferation and migration of HT-29 cells. These findings suggest a molecular mechanism by which dysregulated lipid metabolism and cancer promotion and progression could simultaneously be affected through the use of the same therapeutic tool.

2. Materials and methods

2.1. Cells, adipocyte differentiation and treatments

Human colon adenocarcinoma HT-29 cells were obtained from the American Type Culture Collection (Rockville, MD). HT-29 cells bear a mutated p53 (p.Arg273His), but a wild-type ras allele. These cells were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine. The cells were cultured in 60 mm plates, to 70%–80% confluence, in a humidified atmosphere with 5% CO_2.

3T3-L1 cells were kindly provided by Prof. Beguinot (Department of Translational Medical Sciences, 'Federico II' University of Naples, Italy). To differentiate 3T3-L1 preadipocytes to mature adipocytes, 8.0×10^4 cells were seeded in a 60 mm culture plate. After growth for two days to reach confluency (day 0 [D0]), the preadipocyte expansion medium (DMEM with 10% foetal calf serum [FCS]) was removed and 1-methyl-3-isobutylxanthine(IBMX), dexamethasone, and insulin (MDI)-induced adipocyte differentiation medium was added to the growth-arrested 3T3-L1 preadipocytes. Cells were, indeed, cultured for 48 h with MDI containing DMEM (with 4.5 g/l glucose), 10% FBS, IBMX (0.5 mM), dexamethasone (1 μM) and insulin (1 μg/ml). Starting from day 2 (D2), cells were transferred to and cultured in the adipocyte maintenance medium containing DMEM (4.5 g/l glucose), 10% FBS and 1 μg/ml insulin. This medium was changed every 48 h until day 8 (D8).

Dr. Salvatore Moricca (Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente (DiSPAA), Università degli Studi di Firenze, Piazzale delle Cascine 18, 50144 Firenze, Italy) isolated the fungus *Cladosporium tenuissimum*, while Dr. Sabrina Dallavalle (Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, *via* Celoria 2, 20133 Milano, Italy) purified and sent to us cladosporol A and cladosporol B [22].

Rosiglitazone (RGZ), cladosporol A, cladosporol B and GW9662 were dissolved in dimethyl sulphoxide (DMSO) and mixed with fresh medium to achieve the final concentration. In all treatments, the DMSO final concentration in the medium was less than 0.1%. RGZ, cladosporol A or

cladosporol B (5 μM) were added each time and the medium was replaced except for experiments where it was added only from D0 to D2 or from D2 to D8. We preincubated 3T3-L1 preadipocytes for 3 h with 5 μM GW9662.

2.2. Antibodies and reagents

Antibodies against PPARγ, C/EBPα, p21 and β-catenin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against leptin, adiponectin, cyclin D1, caspase-3, β-actin and cyclophilin were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-mouse and anti-rabbit IgG secondary antibodies conjugated to peroxidase as well as ECL and ECL Plus western blotting detection kits were purchased from Amersham Life Science (Little Chalfont, Buckinghamshire, UK). DMEM, RGZ, GW9662, IBMX, dexamethasone, insulin, Oil Red O and isopropanol were from Sigma Aldrich (St. Louis, MO, USA). FBS, penicillin-streptomycin, L-glutamine and trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Gibco (Carlsbad, CA, USA). Lipofectamine 3000 Reagent was from Invitrogen (Carlsbad, CA, USA).

PPARγ1/2 /E-8) Santa Cruz Biotechnology (Santa Cruz, CA, USA) mw:57 kDa Dilution:1:1000 code: sc-7273.

C/EBPα Santa Cruz Biotechnology (Santa Cruz, CA, USA) mw:42 kDa Dilution: 1:1000.

code: sc-365318.

β-actin Cell Signaling Technologies (Danvers, MA, USA) mw: 45 kDa Dilution 1:1000.

code: #3700.

Leptin Abcam (Cambridge, UK) mw 16 kDa Dilution 1:1000 code: ab3583.

Adiponectin Cell Signaling Technologies (Danvers, MA, USA) mw: 27 kDa Dilution 1:1000 code:#2789.

β-Catenin Santa Cruz Biotechnology (Santa Cruz, CA, USA) mw:105 kDa Dilution 1:1000.

code: sc-7963.

Cyclin D1 Cell Signaling Technologies (Danvers, MA, USA) mw: 36 kDa Dilution 1:1000.

code: #2922.

p-21 Santa Cruz Biotechnology (Santa Cruz, CA, USA) mw: 21 kDa Dilution 1:1000.

code: sc-271610.

Caspase 3 Cell Signaling Technologies (Danvers, MA, USA) mw: 35 kDa Dilution 1:1000.

code: #9662.

2.3. Cell viability

The growth rate of 3T3-L1 cells was evaluated using the MTT assay (Promega, Madison, WI, USA), as previously described [22]. To further evaluate cell growth after treatment with cladosporol A or cladosporol B, cells were plated in 12-well plates at density of 1×10^6 cells/cm^2. After treatment, the cells were washed with phosphate-buffered saline (PBS), trypsinised and collected in culture medium. Cell counting was performed as previously reported [22].

2.4. Western blotting

Treated and untreated cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1% NP-40) containing a protease inhibitor cocktail and then centrifugated at 17,000 *g* for 10 min at 4°C. The protein concentrations of the supernatant was quantified and 80 μg of each sample were separated on 12% acrylamide gels using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was carried out as previously reported [22]. The relative intensity of protein bands was measured using the Molecular Imager Chemi-Doc imaging system and evaluated with ImageLab software (Bio-Rad, Hercules CA, USA) in comparison with the β-actin or cyclofillin used as control. The protein levels of extracellular leptin and adiponectin, reported in Fig. 8B, were normalized in comparison with the total extracellular proteins stained by the Ponceau dye. For each sample the same volume of extracellular total proteins was loaded.

2.5. Oil Red O staining

Oil Red O staining was performed by treating preadipocytes and mature 3T3-L1 adipocytes (after 8 days of differentiation) with 4% formaldehyde for 5 min at room temperature. The cells were washed with 1× PBS, incubated with 60% isopropanol for 5 min and dried completely. Fixed cells were stained with Oil Red O in 60% isopropanol for 1 h at room temperature. Stained lipid droplets in 3T3-L1 cells were imaged with a Nikon Eclipse TS100 microscope. Intracellularly stored lipids were quantified by extracting Oil Red O stain with 100% isopropyl alcohol; the optical density was measured at 500 nm by a Eppendorf BioPhotometer 6131.

2.6. Semiquantitative reverse-transcriptase PCR and real-time PCR

Total RNA was extracted from all experimental points of differentiated and undifferentiated 3T3L1 cells using Trizol reagent according to the manufacturer's instruction (Thermofisher, Whaltam, MA, USA). Total RNA was reverse transcribed to complementary DNA (cDNA) using a random hexadeoxynucleotide primer and reverse transcriptase. Single-stranded cDNA was amplified by PCR using specific primers. The primer sequences, the relative amplicon sizes and Gene ID are as follows:

PPARγ2: 5′-TGGTGCCTTCGCTGATGC-3′ and 5′-CTGTGGTAAAGGGCTTGATGTC-3′;

C/EBPα: 5′-CGACTTCTACGAGGTGGAGC-3′ and 5′-TCGATGTAGGCGCTGATGTC-3′;

aP2: 5′-CACCATCCGGTCAGAGAGTACTTT-3′ and 5′-GCCATGTAGGGTTATGATGCTCTT-3′;

LPL: 5′-GATGCCCTACAAAGTGTTCCATTA-3′ and 5′-CCACTGTGCCGTACAGAGAAAT-3′;

FASN: 5′-GGCTCTATGGATTACCCAAGC-3′ and 5′-CCAGTGTTCGTTCCTCGGA-3′;

GLUT4:5-CTGCTTCTGGCTCTCACAGTACTC-3′ and 5-AGGTTCCGGATGATGTAGAGGTAT-3′;

Adiponectin: 5′-TCCGGGACTCTACTACTTCTCTTACCAC-3′ and 5′GTCCCCATCCCCATACACCTG-3′;

Leptin: 5′-TGACACCAAAACCCTCATCA-3′ and 5′-ATCCAGGCTCTCTGGCTTCT-3′;

Perilipin: 5′- GCTCTTCAATACCCTCCAGAAAAG-3′ and 5′-TTCGAAGGCGGGTAGAGATG-3′.

Cyclophilin was used as internal control; the primer sequences are: 5′-TTCCAGGATTCATGTGCCAG-3′ and 5′-CCATCCAGCCATTCAGTCTT-3′.

PPARG2 Amplicon size 161 bp Gene ID: 19016 NM_011146.3.

cEBPALPHA Amplicon size 195 bp Gene ID: 12606 NM_001287514.1.

AP2 Amplicon Size 147 bp Gene ID:11770 NM_024406.3.

LPL Amplicon Size 107 bp Gene ID: 16956 NM_008509.2.

FASN Amplicon size 190 bp Gene ID: 14104 NM_007988.3.

GLUT 4 Amplicon size 98 bp Gene ID: 20528 NM_001359114.1.

ADIPONECTIN Amplicon Size 199 bp Gene ID: 11450 NM_009605.

LEPTIN Amplicon Size 310 bp Gene ID: 16846 NM_008493.

CYCLOPHILIN (PPIA) Amplicon Size 202 bp Gene ID: 268373 NM_008907.2.

Semiquantitative reverse-transcriptase PCR was carried out by performing 35 cycles (94 °C for 1 min, 54–64 °C for 30 s and 72 °C for 1 min, for each cycle). PCR products were analysed on 2% agarose gels containing ethidium bromide. Gel images were acquired with the Chemi-Doc imaging system (Bio-Rad). To normalize the results cyclophilin

was used as reference gene. For real-time PCR assays, a 20 μl mixture containing 25 ng cDNA, 10 μl SYBR Green and 0.5 μM primers was prepared for each sample. All reactions were prepared using Quantifast SYBR Green PCR kit (Qiagen, Hilden, Germany) and performed according manufacturer's instructions. The assays were run on the CFX96 Real-Time PCR Detection system (Bio-Rad) and evaluated with CFX Manager software. Cyclophilin was used as reference gene for relative quantification in real-time PCR experiments.

2.7. Cell migration (wound healing) assay

The migration ability of the HT-29 cells was investigated using a wound healing assay. Cells were seeded in 12-well plates at a subconfluent density (8×10^4 cells/ml) in growth medium and were allowed to adhere overnight. Once the cells reached 100% confluence, a wound was formed using a plastic micropipette tip, and the conditioned medium from 3T3-L1 cells was used to culture HT-29 cells. Briefly, 3T3-L1 cells were differentiated as described in 2.1 and at day 8 (D8) medium from each different sample (MDI, MDI + RGZ, MDI + ClA, MDI-ClA, MDI + ClB, MDI-ClB) was collected, filtered, concentrated through Amicon centrifugal filter unit Ultra 2 ml 10 K (Millipore Sigma MA, USA) and conserved at −80 °C. This medium was used to evaluate HT-29 cell migration. Cell capacity to migrate was analysed by acquiring images of the scratch after formation (0 h), and 24, 48 and 72 h later. Images were captured using a Samsung Exinos 9 Octa 9820 camera attached to a Nikon TS2 microscope and quantified by Image J software.

2.8. Transient transfection assays

A plasmid bearing the PPRE (PPAR response element) motif for transfection assays in 3T3-L1 cells was a gift of Spiegelman's group. This PPRE-Luc plasmid has a luciferase reporter gene under the transcriptional control of the herpes simplex thymidine kinase (TK) promoter fused to three copies of the PPRE derived from Acyl-CoA oxidase gene. As an internal control for all transient transfection assays, we used the RSV-βGal plasmid, expressing β-galactosidase gene driven by the strong Rous Sarcoma Virus (RSV) promoter. After 2 days of expansion, 3T3-L1 proliferating cells were transfected by Lipofectamine 3000 Reagent in accordance with the manufacturer's instructions and after further 24 h treated with 5 μM RGZ, cladosporol A, cladosporol B, respectively. After 48 h cells were collected, total protein extracts were produced and transactivation activities were evaluated by luciferase assay as previously described [26]. The values were normalized by β-galactosidase assay and the average value for each triplicate was calculated. The same protocol of transfection assay was performed to evaluate the transactivation activities in 3T3-L1 mature adipocytes. After the transfection, preadipocytes were cultured for further two days in differentiation medium (MDI) plus RGZ, cladosporol A, cladosporol B, respectively. 3T3-L1 preadipocytes and mature adipocytes were also pretreated (in the indicated samples of Fig. 5F and G) with 5 μM of GW9662 before the addition of 5 μM RGZ, 5 μM cladosporol A, 5 μM cladosporol B, respectively. Transfection samples were carried out in triplicate and the transactivation activities evaluated by luciferase assay. The values were normalized by β-galactosidase assay and the average value for each triplicate was calculated.

2.9. Statistical procedures

All experiments were performed in duplicate or triplicate with at least two biological replicates and data are expressed as the mean ± standard deviation calculated by Graphpad Prism software. Differences between two groups were assessed using Student's *t*-test. Differences between three or more groups were assessed using a one-way analysis of variance (ANOVA). Asterisks reported in the figure show the degree of statistical significance. A P value <0.05 was considered significant.

3. Results

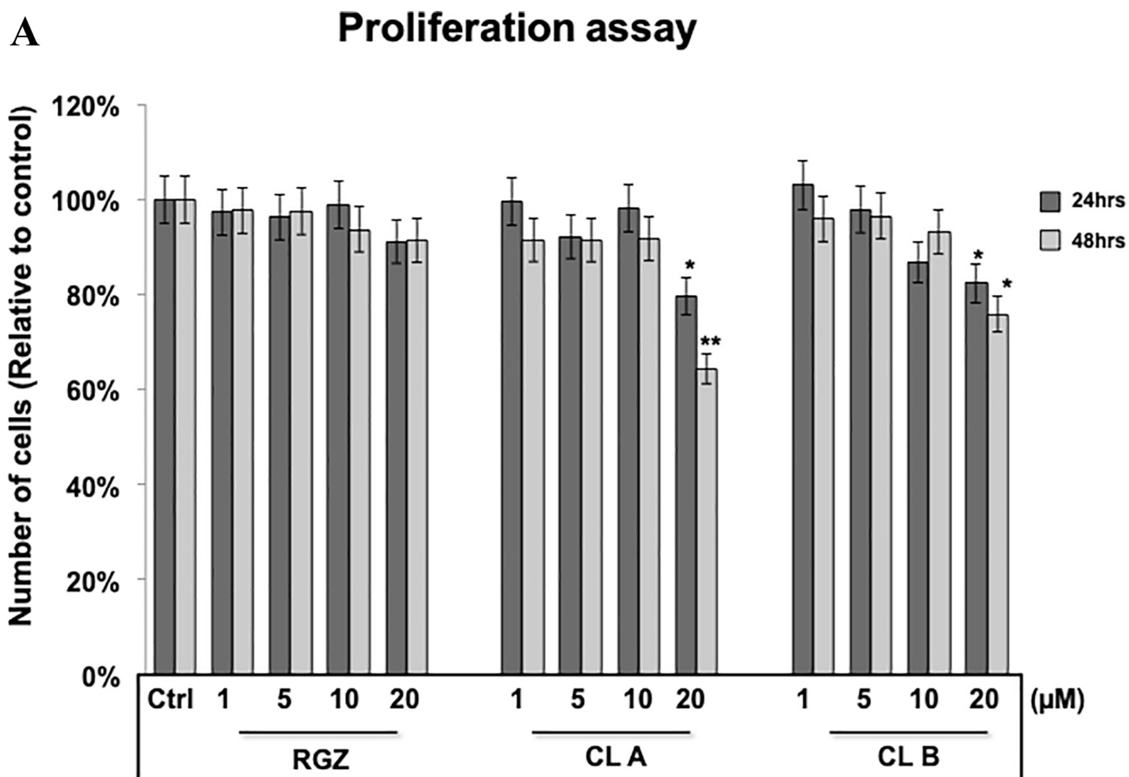
3.1. Cladosporol A and cladosporol B inhibited the in vitro adipogenesis of 3T3-L1 cells

To determine whether cladosporol A and cladosporol B display antiproliferative activity towards 3T3-L1 preadipocytes, we first evaluated the growth of these cells treated for different time points (24 and 48 h) with increasing concentrations of the compounds (1, 5, 10 and 20 μM). There was not a significant reduction in the number of surviving cells after treatment with 1, 5 or 10 μM cladosporol A or cladosporol B for 24 or 48 h (Fig. 1A). There was a partial growth inhibition of 3T3-L1 preadipocytes (about 30% in the presence of cladosporol A and 20% in the presence of cladosporol B) only at the highest concentration (20 μM) and mostly after 48 h exposure. The same treatment times and doses of rosiglitazone (RGZ), a well-known full agonist of PPARγ, did not appreciably reduce the number of 3T3-L1 preadipocytes. To further confirm these data, we subsequently treated 3T3-L1 preadipocytes as described above and evaluated the cytotoxic potential of cladosporol A and cladosporol B using the MTT assay. There was a decrease in 3T3-L1 preadipocyte viability only after exposure to the highest concentration (20 μM) of cladosporol A (about 20% and 33% after 24 and 48 h, respectively) and cladosporol B (about 17% and 23% after 24 and 48 h, respectively) (Fig. 1B). The same doses of RGZ did not affect the 3T3-L1 preadipocyte viability. We previously demonstrated that exposure of HT-29 cells to 10 μM cladosporol B for 48 h inhibited cell proliferation by about 90% [26]. In this study, the administration of cladosporol A or cladosporol B at concentrations below 20 μM did not affect the proliferation of 3T3-L1 preadipocytes. On basis of these data, unless specifically indicated, we used doses ≤10 μM cladosporol A or cladosporol B in the subsequent assays.

We next investigated 3T3-L1 preadipocyte differentiation. The cells were treated with MDI plus 5 μM RGZ, cladosporol A or cladosporol B, respectively (see Fig. 2A) and then stained with Oil Red O after 10 days of differentiation. As shown in Fig. 2B and D, accumulation of lipid droplets was strongly evident in cells treated with 1-methyl-3-isobutylxanthine(IBMX), dexamethasone, and insulin (MDI) or MDI plus RGZ, thus suggesting that morphological and functional changes were associated with differentiation of 3T3-L1 preadipocytes to mature adipocytes. On the contrary, exposure to MDI plus cladosporol A or cladosporol B appeared to inhibit lipid accumulation, thus indicating that both molecules impaired adipogenesis. Fig. 2E shows the quantitative evaluation of the Oil Red O–stained cells through spectrophotometric analysis at 500 nm. Treatment with MDI plus 5 μM cladosporol A or cladosporol B inhibited lipid storage in the mature adipocytes compared with treatment with MDI or MDI plus RGZ. Based on the viability and differentiation data (Figs. 1 and 2), we can confirm that the antiadipogenic activity of both cladosporol A and cladosporol B is not dependent on a general cytotoxicity.

3.2. Cladosporol A and cladosporol B downregulated the expression of adipogenesis-specific genes in 3T3-L1 cells

To clarify the molecular mechanisms underlying the inhibitory effects of cladosporol A and cladosporol B on adipogenesis in 3T3-L1 cells, we performed semiquantitative reverse-transcriptase PCR on total mRNA extracted from 3T3-L1 preadipocytes after 3 and 10 days of treatment with MDI plus 5 μM RGZ or 5 μM cladosporol A or 5 μM cladosporol B, respectively. PPARγ and C/EBPα mRNA levels were significantly increased during the early stages of adipogenesis (within 3 days) when treated with MDI or MDI plus RGZ (Fig. 3A). Administration of MDI plus cladosporol A or cladosporol B, on the contrary, caused a noticeable reduction in both of these early adipogenic markers. After 10 days of differentiation, although the basal PPARγ2 and C/EBPα mRNA levels appeared to be quite different, exposure of the mature adipocytes to cladosporol A or cladosporol B also induced an evident reduction in



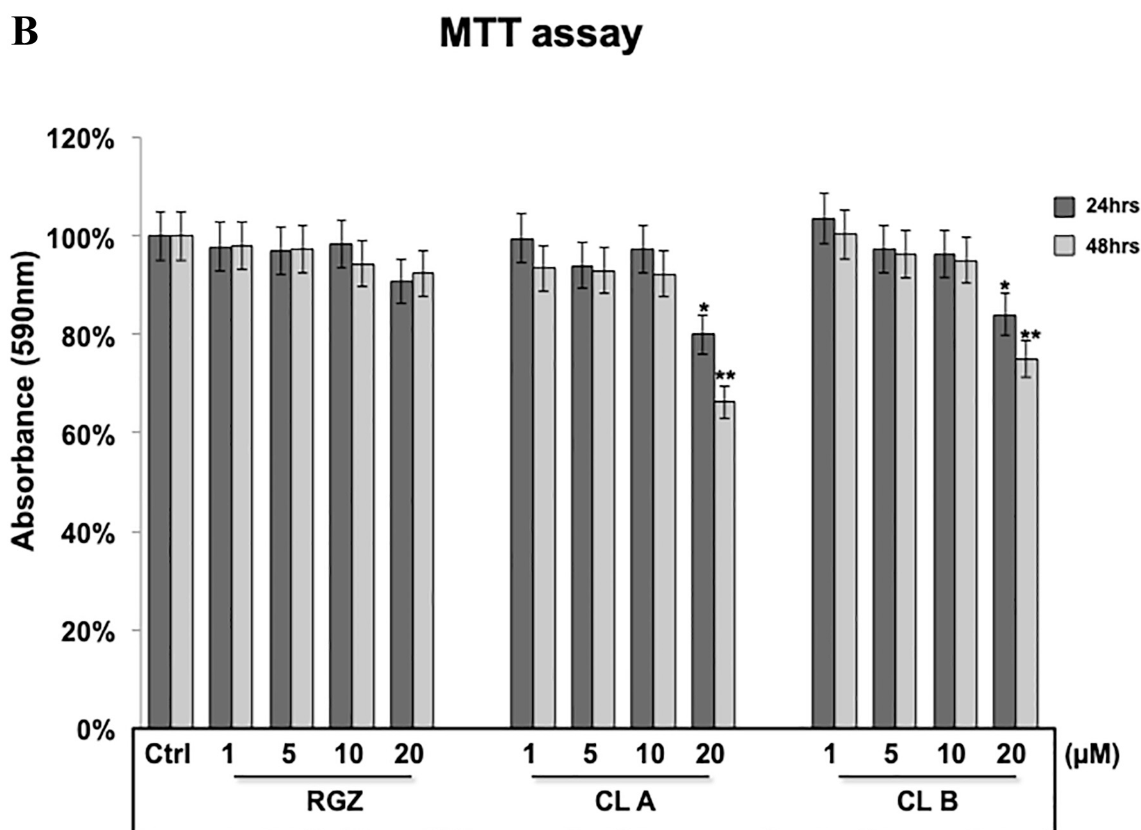


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Fig. 1. Treatment with cladosporol A or cladosporol B did not affect 3T3-L1 preadipocyte proliferation. (A) The antiproliferative effects of rosiglitazone (RGZ), cladosporol A (CL A) and cladosporol B (CL B) on exponentially growing 3T3-L1 cells: cells were treated or not treated with 1, 5, 10 and 20 μM of the compounds for 24 and 48 h, collected and counted. The data are presented as the mean ± standard deviation of three experiments performed in duplicate. The results were similar in two independent experiments. $*P < 0.05$ and $**P < 0.01$, compared with control (Ctrl), the untreated preadipocytes. (B) Dose-dependent 3T3-L1 cytotoxicity caused by RGZ, CL A and CL B, as determined by the MTT assay. Cells were seeded in 96-well plates at a density of 6.0×10^4 cells/cm^2 in standard conditions. After 24 h, cells were exposed to increasing concentrations of RGZ, CL A or CL B (from 1 to 20 μM) and the growth was evaluated up to 24 and 48 h by measuring the absorbance at 590 nm. The results are expressed as the optical density ratio of the difference between treated and control cells. Each point was performed in triplicate and the experiment was repeated two times. $*P < 0.05$ and $**P < 0.01$, compared with Ctrl, the untreated preadipocytes.

both markers. PPARγ2 and C/EBPα play a central role in inducing adipogenesis because their overexpression positively regulates genes required for the terminal differentiation phase of adipogenesis, such as FASN, aP2, LPL and GLUT4. Although the basal levels of these terminal differentiation markers of functional adipocytes are different, their expression was clearly affected by the administration of MDI plus either cladosporol compared with the cells treated with MDI or MDI plus RGZ (Fig. 3A). Mature adipocytes usually secrete several endocrine signals to participate in caloric homeostasis. Thus, we next examined whether leptin and adiponectin expression was affected by the administration of cladosporol A or cladosporol B during the terminal differentiation of 3T3-L1 cells. Leptin and adiponectin mRNA levels in 3T3-L1 mature adipocytes were significantly decreased after treatment with cladosporol A or cladosporol B, indicating a strong inhibition of adipogenesis (Fig. 3A).

We confirmed the semiquantitative data with real-time PCR, using total RNA extracted from 3T3-L1 adipocytes. As expected, expression of the two early adipogenesis-related genes, PPARγ and C/EBPα, was significantly reduced after treatment with MDI plus 5 μM cladosporol A or cladosporol B for 3 days (Fig. 3B). Furthermore, the mRNA level of four of terminal differentiation markers (aP2, FASN, adiponectin and perilipin) was diminished when the cells were treated with MDI plus 5 μM cladosporol A or cladosporol B for 10 days, compared with cells treated with MDI or MDI plus RGZ (Fig. 3C).

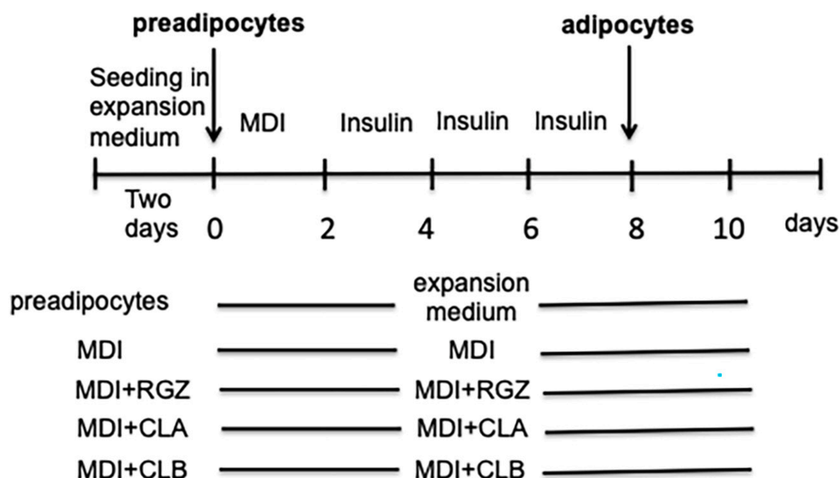
We also analysed by western blotting the protein levels of the same markers for which we examined transcript levels. The two early markers, PPARγ and C/EBPα (Fig. 4A), and the two terminal differentiation markers, leptin and adiponectin (Fig. 4B), were downregulated in the presence of MDI plus 5 μM cladosporol A or cladosporol B compared with cells treated with MDI or MDI plus RGZ. Our gene and protein expression results suggest that cladosporol A and cladosporol B inhibited adipogenesis in 3T3-L1 cells by reducing the mRNA and protein levels of early and terminal differentiation markers of adipogenesis.

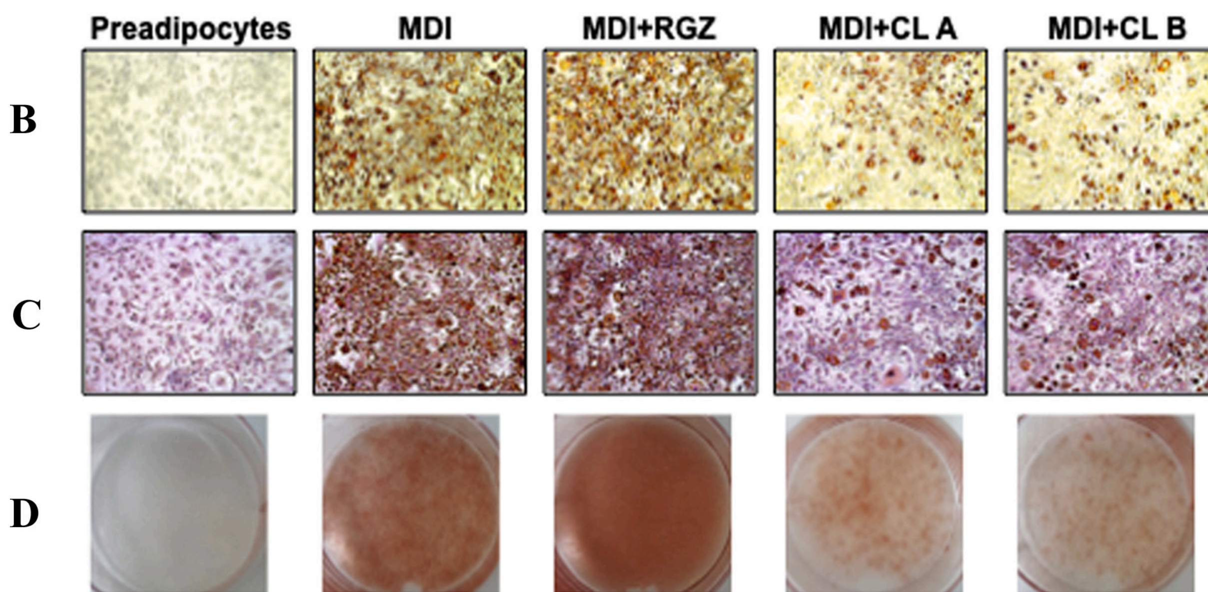
3.3. *Cladosporol A and cladosporol B displayed PPARγ-mediated antiadipogenic activity*

To demonstrate definitively that PPARγ mediates the antiadipogenic signals of the cladosporols, we preincubated 3T3-L1 preadipocytes for 3 h with 5 μM GW9662, a well-known irreversible inhibitor of PPARγ, and subsequently treated cells with MDI or MDI plus 5 μM RGZ, cladosporol A or cladosporol B, respectively. We allowed the cells to differentiate until up to 10 days and then stained them with Oil Red O to evaluate the accumulation of intracellular lipids. Results of Fig. 5A demonstrated that preincubation of 3T3-L1 cells with 5 μM GW9662 caused a significant decrease of lipid depots in all the tested conditions (MDI, MDI plus RGZ, MDI plus cladosporol A and MDI plus cladosporol B), suggesting that this specific irreversible inhibitor subtracted a part of the available and functional PPARγ. Data from Fig. 5B, showing the quantitative evaluation of the Oil Red O–stained cells through spectrophotometric analysis at 500 nm, confirmed the diminished ability of the mature adipocytes to accumulate lipids when the cells were pretreated with GW9662. To verify that pretreatment with GW9662 impaired the binding to PPARγ of the other ligands and, in such way, triggered the receptor degradation, we preincubated 3T3-L1 preadipocytes with 5 μM GW9662 and analysed by western blotting PPARγ expression in cells successively exposed to MDI, MDI plus 5 μM RGZ, MDI plus 5 μM cladosporol A and MDI plus 5 μM cladosporol B. Results from Fig. 5C demonstrated that GW9662 pretreatment caused a reduction in PPARγ expression in all tested conditions. These data suggested that this inhibitor, that covalently binds to the PPARγ ligand binding domain (LBD) pocket, may sequester functional and available receptor so that it cannot bind to the tested ligands (RGZ, cladosporol A and cladosporol B). Moreover, it is remarkable that expression of both PPARγ1 and PPARγ2 (the adipogenic specific isoform containing about 30 additional amino acids at the N-terminus) was affected by GW9662 binding, thus preventing the specific interaction with the tested ligands (RGZ, cladosporol A and cladosporol B). In order to further confirm that cladosporol action was mediated by the binding to PPARγ, we transiently transfected 3T3-L1 preadipocytes with a plasmid bearing the PPRE (PPAR response element) motif cloned upstream the basal TK promoter that, in turn, drives the transcription of luciferase reporter gene. As shown in Fig. 5D, the transcriptional activity, promoted by the PPRE motif cloned upstream the luciferase gene, was induced by the canonical agonist of PPARγ, RGZ, whereas was inhibited by both cladosporols. At the same time, we transfected another aliquot of 3T3-L1 preadipocytes and, afterwards, allowed these cells to differentiate to mature adipocytes by MDI treatment. The results of Fig. 5E indicated that the transcription of luciferase reporter gene, driven by PPRE motif cloned upstream the basal TK promoter, was further increased by RGZ treatment and again inhibited by both cladosporols. Note the maior increase (2.0 *vs* 1.5 of arbitrary units) of luciferase gene transcription due to RGZ, when the level of PPARγ expression was induced after at least two days of differentiation through MDI treatment (compare sample 3 of Fig. 5D with sample 3 of Fig. 5E). To demonstrate that binding of cladosporols to PPARγ is the unique condition to permit their action, we repeated the transient transfection assays in preadipocytes (Fig. 5F) and in preadipocytes that were differentiated to mature adipocytes (Fig. 5G) in presence and absence of GW9662. Data from these experiments suggested that 5 μM GW9662 pretreatment of both preadipocytes and mature adipocytes caused a reduction of the transcription activity driven by the PPRE motif in all tested conditions (MDI, MDI plus RGZ, MDI plus cladosporol A and MDI plus cladosporol B). Altogether the experiments of Fig. 5 indicate that binding of cladosporols to PPARγ, the master regulatory gene of adipogenesis, is a crucial part of the mechanism by which they inhibit adipogenesis in 3 T3-L1 cells.

3.4. *The antiadipogenic activity of cladosporols A and B influenced the proliferation and invasion of HT-29 cells*

Because adipose tissue plays a prominent role in the tumour microenvironment, we decided to investigate whether secreted factors (adipokines) from 3T3-L1 cells could influence HT-29 cell growth, invasion and migration. Hence, we allowed 3T3-L1 preadipocytes to differentiate (see section 2.1) and collected the medium from cells treated with MDI, MDI plus 5 μM RGZ, 5 μM cladosporol A or 5 μM cladosporol B, respectively. The medium was filtered and used as conditioned medium to examine the effect on HT-29 cell proliferation. The conditioned medium collected from 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B inhibited HT-29 cell proliferation. On the contrary, the conditioned medium from 3T3-L1 treated with only MDI or (to a greater extent) from undifferentiated preadipocytes appeared to permit cell proliferation. The inhibitory effects due to the conditioned medium from 3T3-L1 treated with MDI plus both cladosporols were already evident





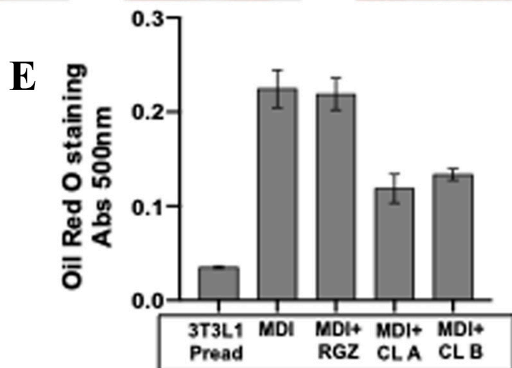


Fig. 2. Cladosporol A and cladosporol B inhibited 3T3-L1 differentiation. (A) Scheme of 3T3-L1 preadipocyte differentiation and cladosporol treatments. (B) Cells were treated for 10 days with the differentiation mixture (MDI), MDI plus 5 μM rosiglitazone (MDI + RGZ) or MDI plus 5 μM cladosporol A (MDI + CL A) or MDI plus 5 μM cladosporol B (MDI + CL B) and then stained with Oil Red O. (C) Cells were also stained with hematoxylin to visualise the nuclei. (D) Photographs of the same cells in the Petri dishes treated as described in (B). (E) The graph shows the quantitative evaluation of Oil Red O staining. The data are presented as the mean ± standard deviation of two different experiments performed in duplicate. The untreated preadipocytes were the control cells.

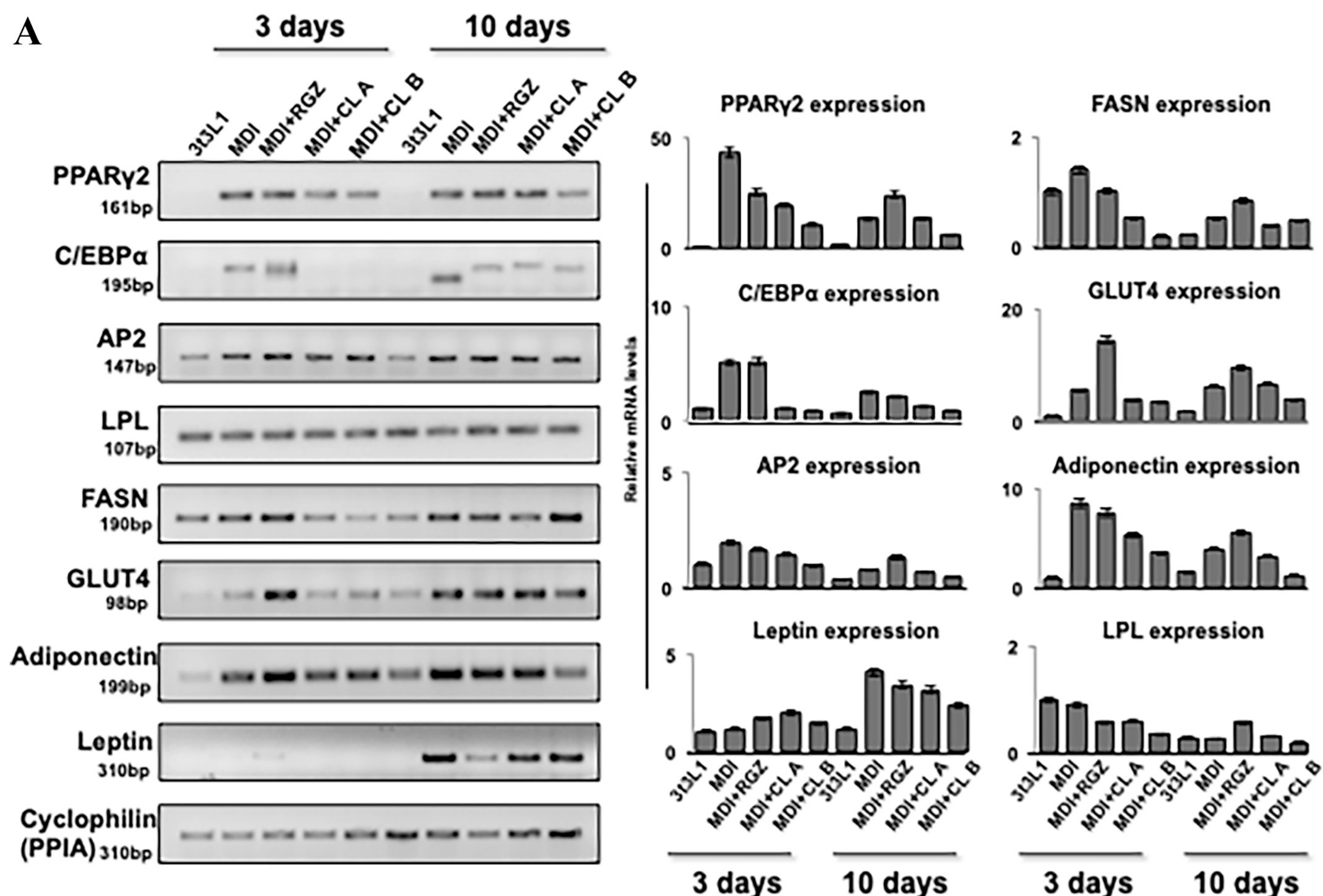


Fig. 3. Cladosporol A and cladosporol B downregulated the expression of adipogenesis-related genes in 3T3-L1 cells. (A) Semiquantitative reverse-transcriptase polymerase chain reaction (PCR) analysis of early (3 days) and late (10 days) adipogenic markers in 3T3-L1 cells. The template for complementary DNA (cDNA) was total RNA extracted from cells treated with the differentiation mixture (MDI), MDI plus 5 μM rosiglitazone (MDI + RGZ) or MDI plus 5 μM cladosporol A (MDI + CL A) or MDI plus 5 μM cladosporol B (MDI + CL B). The untreated preadipocytes (3T3-L1) were the control cells.The expression values were normalized using as control the cyclophilin gene product. The data are presented as the mean ± standard deviation of two different experiments performed in duplicate. (B) Real-time PCR analysis of mRNA levels of early adipogenesis-related genes (PPARγ and C/EBPα) in 3T3-L1 cells. The template for complementary DNA was total RNA extracted from cells treated with MDI, MDI plus 5 μM rosiglitazone (MDI + RGZ) or MDI plus 5 μM cladosporol A (MDI + CL A) or MDI plus 5 μM cladosporol B (MDI + CL B). The expression values were normalized using as control the cyclophilin gene product. The data are presented as the mean ± standard deviation of three experiments performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with Ctrl (3T3-L1). (C) Real-time PCR analysis of mRNA levels of late adipogenesis-related genes (aP2, FASN, adiponectin and perilipin) in 3T3-L1 cells. The details are the same as described in (B). $^*P < 0.05$, compared with Ctrl (3T3-L1).

after 48 h (Fig. 6A), while the reduction in the number of HT-29 cells was even more dramatic after 72 h (Fig. 6B).

To determine the effects of 3T3-L1 conditioned medium on HT-29 cell migration, we carried out a wound healing assay (see details in section 2.6; Fig. 7). We captured images at 0, 48 and 72 h after wound formation to determine the degree of healing. Conditioned medium from 3T3-L1 preadipocytes and differentiated adipocytes (treated with MDI) significantly enhanced the migratory ability of HT-29 cells. On the contrary, migration of the cells was significantly inhibited after exposure to conditioned medium collected from 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B (Fig. 7A). The migratory ability of cells was only partially affected by the conditioned medium collected from 3T3-L1 cells treated with MDI plus RGZ. We repeated the above described experiment, modifying the protocol as indicated in Fig. 7B. The conditioned medium collected from 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B was deprived of both drugs during the last two days of cell differentiation (the new samples are denoted as MDI-CL A and MDI-CL B in Fig. 7B and C). We deemed this protocol variation necessary to determine whether the inhibition of cell migration could be attributed to the effects of cladosporols on 3T3-L1 adipocyte differentiation and not directly to the presence of the drugs in the conditioned medium. In these new experimental conditions, conditioned medium from 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B provided the same inhibition of HT-29 proliferation and migration (Fig. 7C). To demonstrate definitively the inhibitory effects on HT-29 cell growth and migration, we used western blotting assay of the total protein extracted from HT-29 cells treated with the medium from preadipocytes, 3T3-L1 treated with MDI or 3T3-L1 treated with MDI plus RGZ, cladosporol A or cladosporol B, respectively. There was an evident reduction in cyclin D1 and β-catenin and a simultaneous increase in p21 expression, thus suggesting a strong blockage of cell cycle of HT-29 cells grown with the conditioned medium from cells exposed to either cladosporol (Fig. 7D). Moreover, there was a significant stimulation of apoptosis: the same conditioned medium from cells exposed to either cladosporol reduced caspase-3 precursor levels, one of the terminal effectors of programmed cellular death.

Leptin and adiponectin are two adipokines produced by mature adipose tissue to integrate peripheral and central signals, but they also represent components of the milieu that stimulate the tumour microenvironment and thus regulate signaling pathways related to cancer

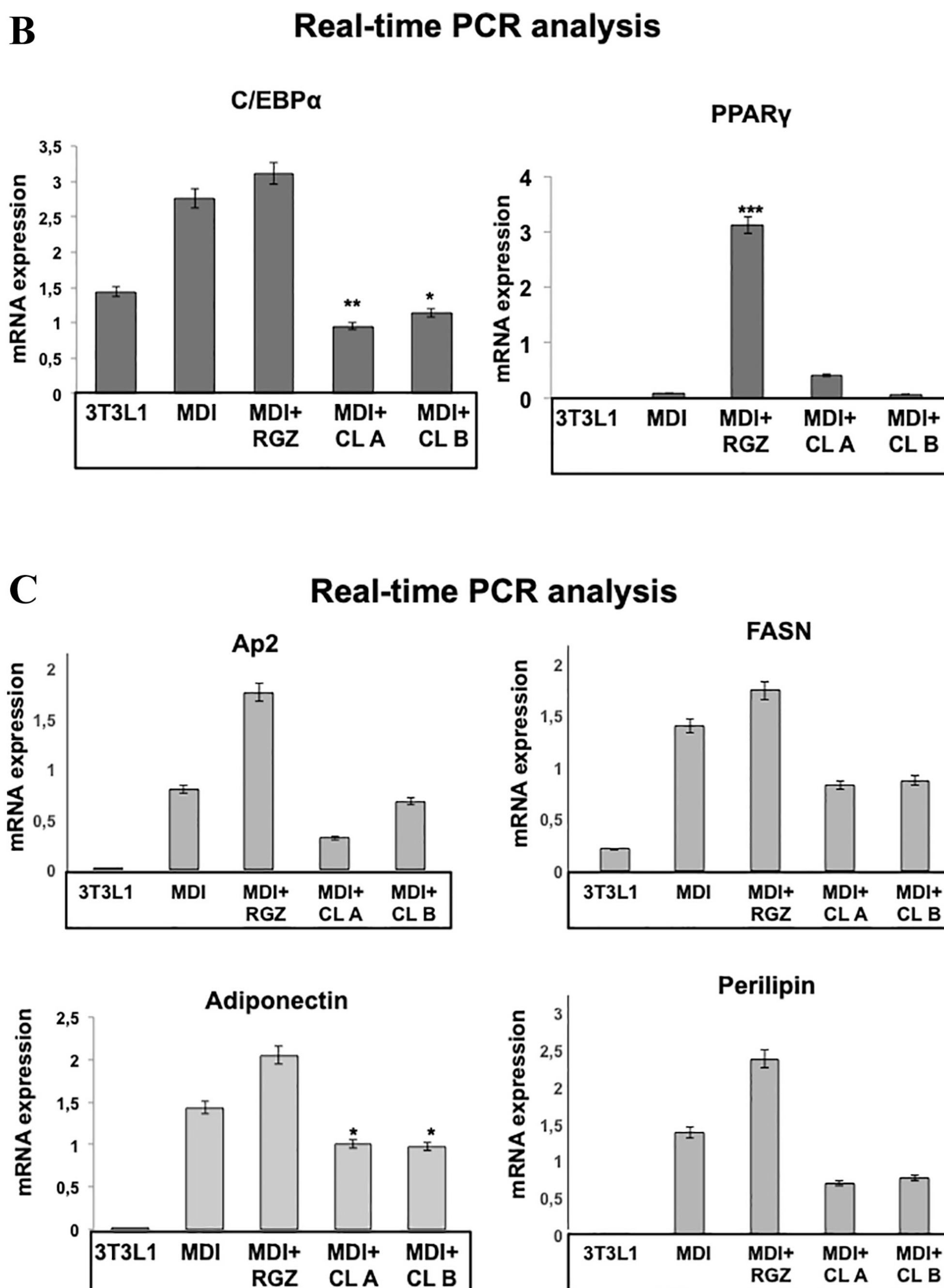


Fig. 3. (*continued*).

progression [27–30]. Therefore, we analysed the presence of these important adipokines in 3T3-L1 cells and in the culture medium of undifferentiated and differentiated 3T3-L1 cells treated with MDI plus RGZ, cladosporol A or cladosporol B, respectively. Both cladosporols reduced the leptin expression level in cells and in the medium (Fig. 8A and B). On the contrary, leptin expression was higher in the 3T3-L1 preadipocytes as well as in the medium from differentiated 3T3-L1 cells treated with MDI or MDI with RGZ. Both cladosporols reduced adiponectin expression in the cells but stimulated adiponectin secretion in the medium (Fig. 8A and B). Collectively, these results strongly suggest that cladosporols are able to regulate production and availability of the most important hormones that differentiated adipocytes secrete in order to influence biological properties of neighbouring cells.

4. Discussion

Obesity, insulin resistance, type 2 diabetes and cardiovascular diseases constitute the most frequent pathological conditions associated with metabolic syndrome, an epidemic that affects a quarter of the world's population. Excessive nutrient intake and very low calorie expenditure due to a limited physical activity appear to be the possible causes of the impressive spread of metabolic syndrome all over the world [31]. Therefore, metabolic syndrome can be considered the result of an imbalance between the storage of energy fuels and their utilisation. Distinctive clinical signs of metabolic syndrome are abdominal obesity, hypertension, dyslipidaemia, insulin resistance and a proinflammatory state [32]. The most recent findings have demonstrated that patients affected by metabolic syndrome can also develop – in addition to cardiovascular diseases, diabetes and obesity – fatty liver, cholesterol

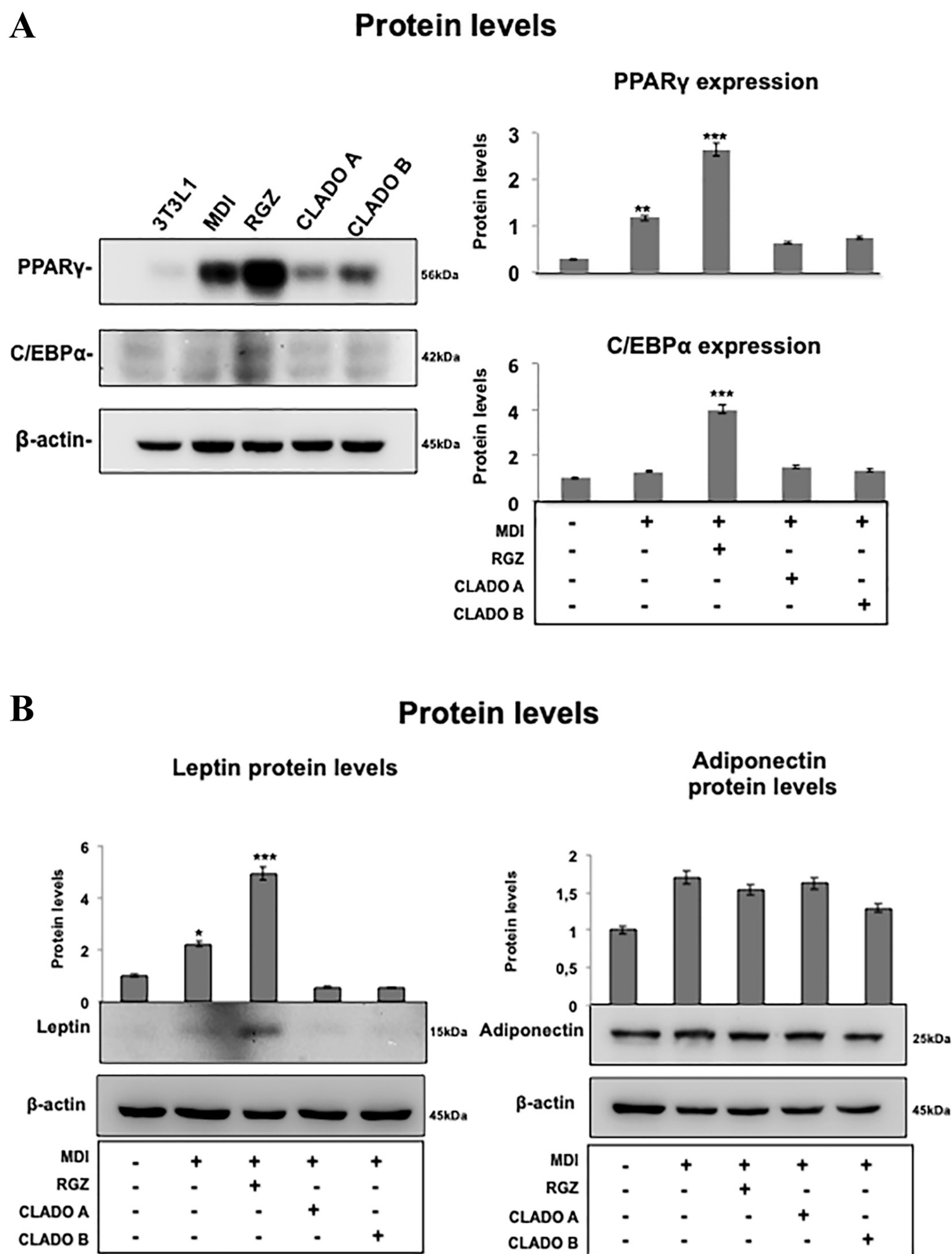


Fig. 4. Cladosporol A and cladosporol B downregulated the expression of adipogenesis-related proteins in 3T3-L1 cells. (A) Western blotting analysis of early adipogenesis-related proteins in 3T3-L1 cells. Total protein from 3T3-L1 cells treated with differentiation mix (MDI) or MDI plus 5 μM rosiglitazone (RGZ) or 5 μM cladosporol A (CLADO A) or 5 μM cladosporol B (CLADO B) were separated by sodium dodecyl sulfate–polyacrylamide gel electropheresis and transferred to nylon membranes. Subsequently, antibodies were used to detect the expression of PPARγ and C/EBPα. The left part of figure shows a representative experiment, whereas the right part of the figure shows the quantitative evaluation of protein levels of both markers. To control the samples loaded derived from untreated and cladosporol A- and cladosporol B-treated 3T3-L1 cells and normalize the results, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of protein/β-actin of at least 3 independent experiments **$P < 0.01$ and ***$P < 0.001$, compared with control (3T3-L1). (B) Western blotting analysis of of late adipogenesis-related proteins in 3T3-L1 cells. Description of the assay is as in (A). Specific antibodies were used to detect the expression of leptin and adiponectin. The details of the evaluation also are the same as described in (A). *$P < 0.05$ and ***$P < 0.001$, compared with control (3T3-L1).

gallstones and some types of cancer, including pancreatic, breast, bladder and colorectal [33]. To manage efficiently not only cancer, but also metabolic syndrome and its related consequences (obesity, hypertension, dyslipidaemia, insulin resistance and cardiovascular diseases), a dual approach has been proposed: lifestyle modification (stimulating physical activity and diminishing food intake) and efficient chemoprevention using natural and/or synthetic compounds. This dual intervention should ameliorate systemic metabolic dysfunctions by targeting insulin resistance, obesity, hypertension, hyperglycaemia, dyslipidaemia and inflammation, thus simultaneously reducing several risk factors

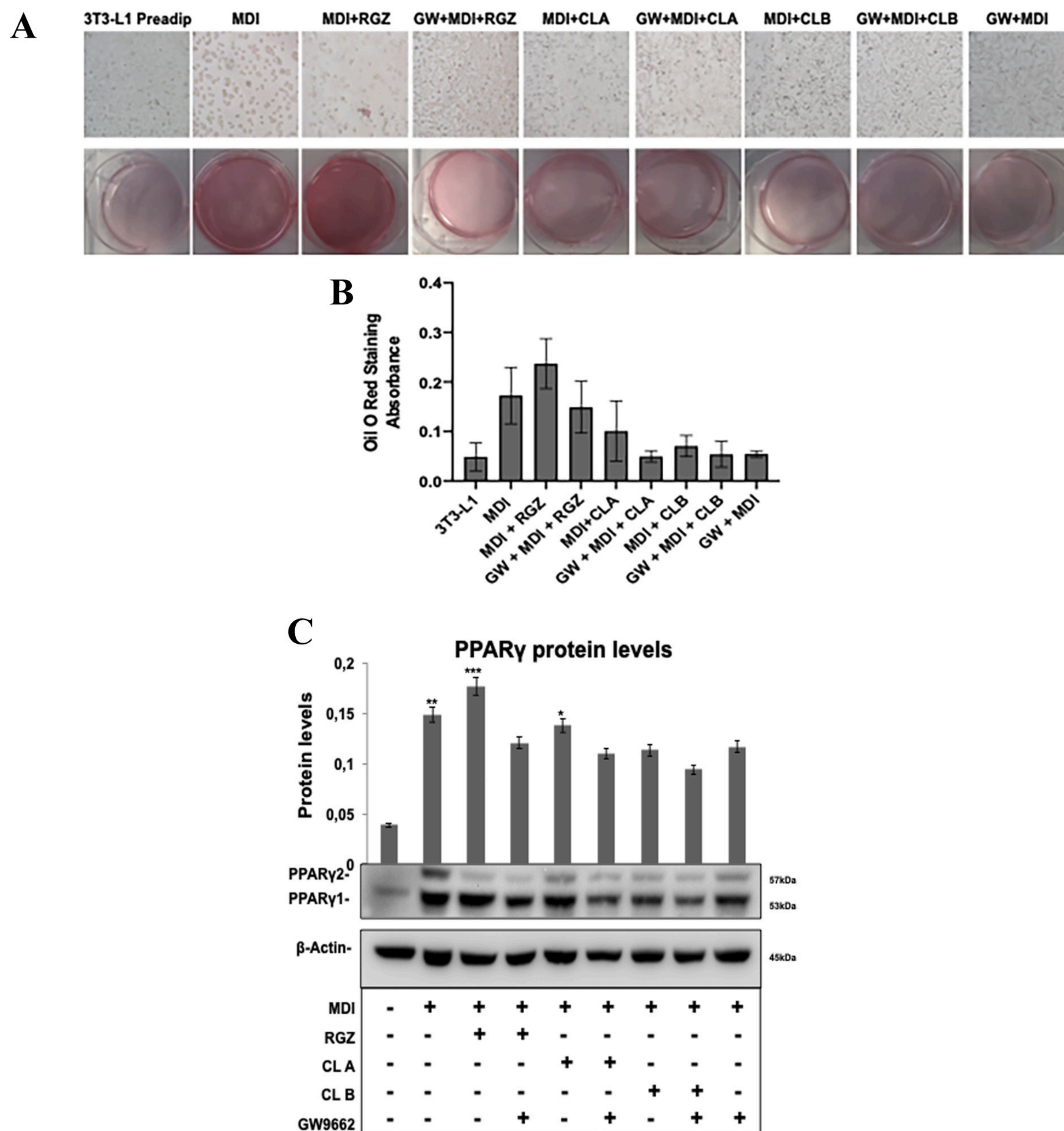
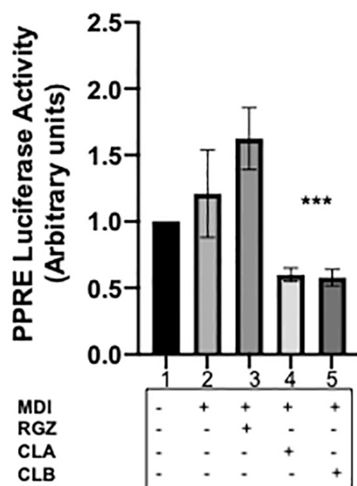
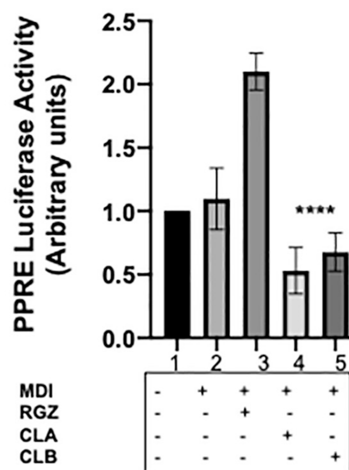
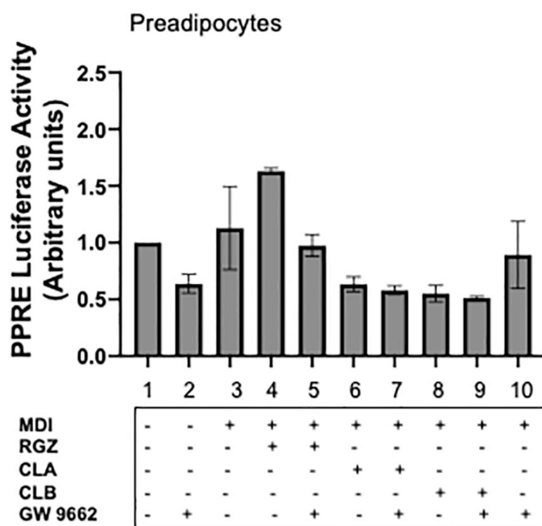


Fig. 5. PPARγ mediated the antiadipogenic effect of cladosporols. (A) 3T3-L1 cells, after 2 days of expansion, were firstly exposed for three hours to GW9662 (5 μM) and then treated for 10 days with the differentiation mixture MDI (GW + MDI), MDI plus 5 μM rosiglitazone (GW + MDI + RGZ) or MDI plus 5 μM cladosporol A (GW + MDI + CL A) or MDI plus 5 μM cladosporol B (GW + MDI + CL B). Samples from no pretreated cells with GW9662 are denoted with MDI, MDI + RGZ, MDI + CL A, MDI + CL B. Successively, they were stained with Oil Red O. Photographs of the same cells in the Petri dishes treated as described in (A) are also shown. (B) The graph shows the quantitative evaluation of Oil Red O staining. The data are presented as the mean ± standard deviation of two different experiments performed in duplicate. The untreated preadipocytes 3T3-L1 were the control cells. (C) Western blotting analysis of PPARγ1 and PPARγ2 protein levels. Total protein was extracted from untreated 3T3-L1 cells and 3T3-L1 cells treated with differentiation mix (MDI) or MDI plus rosiglitazone (RGZ), cladosporol A (CL A) or cladosporol B (CL B), in the presence or absence of GW9662. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nylon membranes. Subsequently, antibodies were used to detect the expression of PPARγ1 and PPARγ2. To control the samples loaded derived from untreated and cladosporol A- and cladosporol B-treated 3T3-L1 cells and normalize the results, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of protein/β-actin of at least 3 independent experiments.*P < 0.05, **P < 0.01 and ***P < 0.001, compared with control (Ctrl). (D) Transient transfection assay performed in 3T3-L1 preadipocytes. A plasmid bearing the PPRE (PPAR response element) motif cloned upstream the basal TK promoter that, in turn, drives the transcription of luciferase reporter gene was transfected in 3T3-L1 proliferating cells and, subsequently, treated with RGZ, cladosporol A, cladosporol B, respectively. The data are presented as the mean ± standard deviation of two different experiments performed in triplicate. ***$P < 0.0002$, compared with transfected but non treated cells used as control (sample 1 of Fig. 5D). (E) Transient transfection assay performed in 3T3-L1 mature adipocytes. The same protocol of transfection assay was performed, but the preadipocytes, after the transfection, were cultured in differentiation medium (in presence of MDI) plus RGZ, cladosporol A, cladosporol B, respectively. The data are presented as the mean ± standard deviation of two different experiments performed in triplicate. ****$P < 0.0001$, compared with transfected but non treated cells used as control (sample 1 of Fig. 5E). (F) Transient transfection assay performed in 3T3-L1 preadipocytes in presence and absence of GW9662. The protocol of transfection assay was the same of (D), but the transfected preadipocytes were firstly pretreated for 3 h with 5 μM of GW9662 and then exposed to the differentiation mixture (MDI) plus 5 μM rosiglitazone (RGZ) or 5 μM cladosporol A (CL A) or 5 μM cladosporol B (CL B). The data are presented as the mean ± standard deviation of

two different experiments performed in duplicate. The data were normalized with transfected but non treated cells used as control (sample 1 of Fig. 5F). (G) Transient transfection assay performed in 3T3-L1 mature adipocytes in presence and absence of GW9662. The protocol of transfection assay was the same of (E), but the transfected preadipocytes were firstly pretreated for 3 h with 5 μM of GW9662 and then exposed to the differentiation mixture (MDI) plus 5 μM rosiglitazone (RGZ) or 5 μM cladosporol A (CL A) or 5 μM cladosporol B (CL B). The data are presented as the mean ± standard deviation of two different experiments performed in duplicate. The data were normalized with transfected but non treated cells used as control (sample 1 of Fig. 5G).







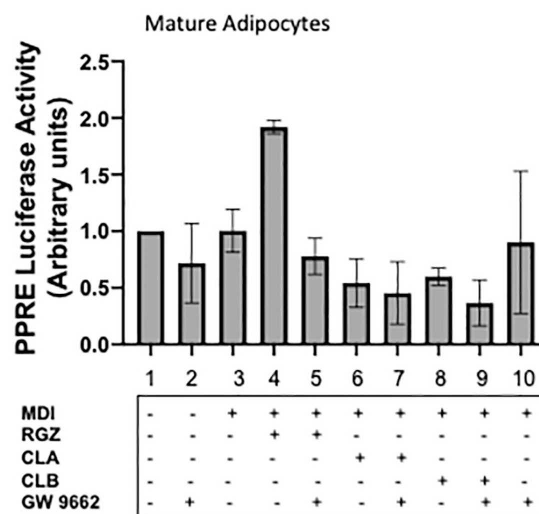


Fig. 5. (*continued*).

of cancer.

In recent studies, we focused on the role of cladosporols, secondary metabolites derived from *C. tenuissimum*, in controlling CRC cell proliferation [22,23,26]. Cladosporols exert this function by acting as ligands of PPARγ, one of the PPAR isoforms. PPARs, which are type II nuclear receptors, regulate a wide range of biological processes, including glucose and lipid metabolism, differentiation, cell growth and inflammation, in different tissues. A relationship between adipogenesis and cancer progression has been demonstrated [34]; hence, we wanted to investigate whether cladosporols, as PPARγ ligands that interfere with two relevant pathological processes – lipid dysregulation and increased cell proliferation – could be efficient therapeutic tools to control metabolism and cancer and thus simultaneously prevent obesity and cancer. Cladosporol A and cladosporol B inhibited adipogenesis in 3T3-L1 cells, reducing accumulation of fatty acids that are then transformed to triglycerides in mature adipocytes (Fig. 2). Both cladosporols interfered with adipogenesis, denoted by decreased mRNA and protein expression of early and late adipogenic markers (Figs. 3 and 4). Among these differentiation markers, PPARγ plays a crucial, master regulatory role in stimulating adipogenesis, and the inhibitory effect promoted by both cladosporols on PPARγ mRNA and protein expression is dramatic (Figs. 3A, Figs. 3B and 4A). Western blotting unequivocally confirmed that cladosporol A and cladosporol B activity in 3T3-L1 cells depends on binding to PPARγ. Indeed, pretreatment of these cells with GW9662, an irreversible inhibitor of PPARγ, reduced the expression of both isoforms (PPARγ1 and PPARγ2) in cells treated with cladosporol A or cladosporol B thus causing also an inhibitory effect on the 3T3-L1 cell differentiation and lipid accumulation (Fig. 5A, B and C). Transient transfection assays,

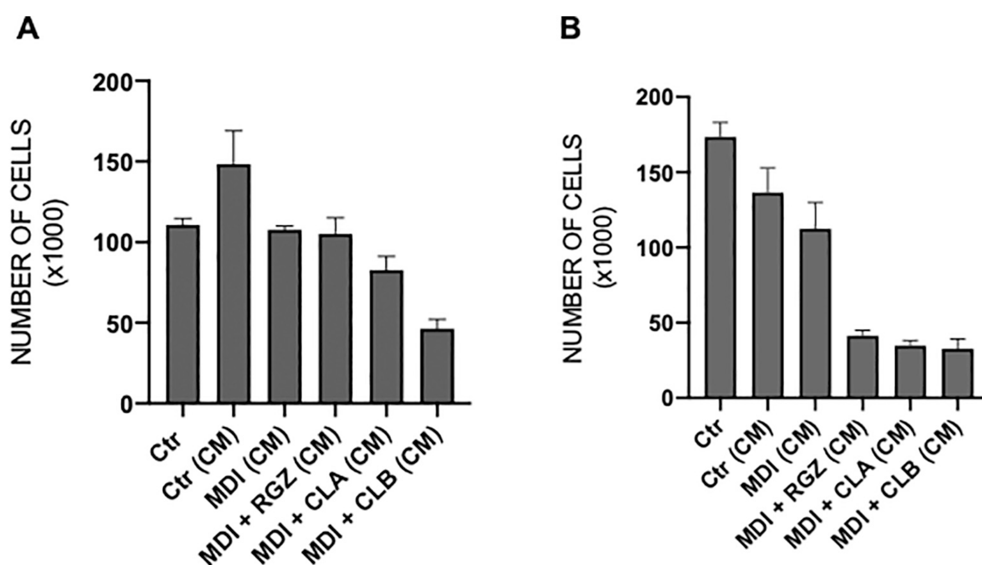


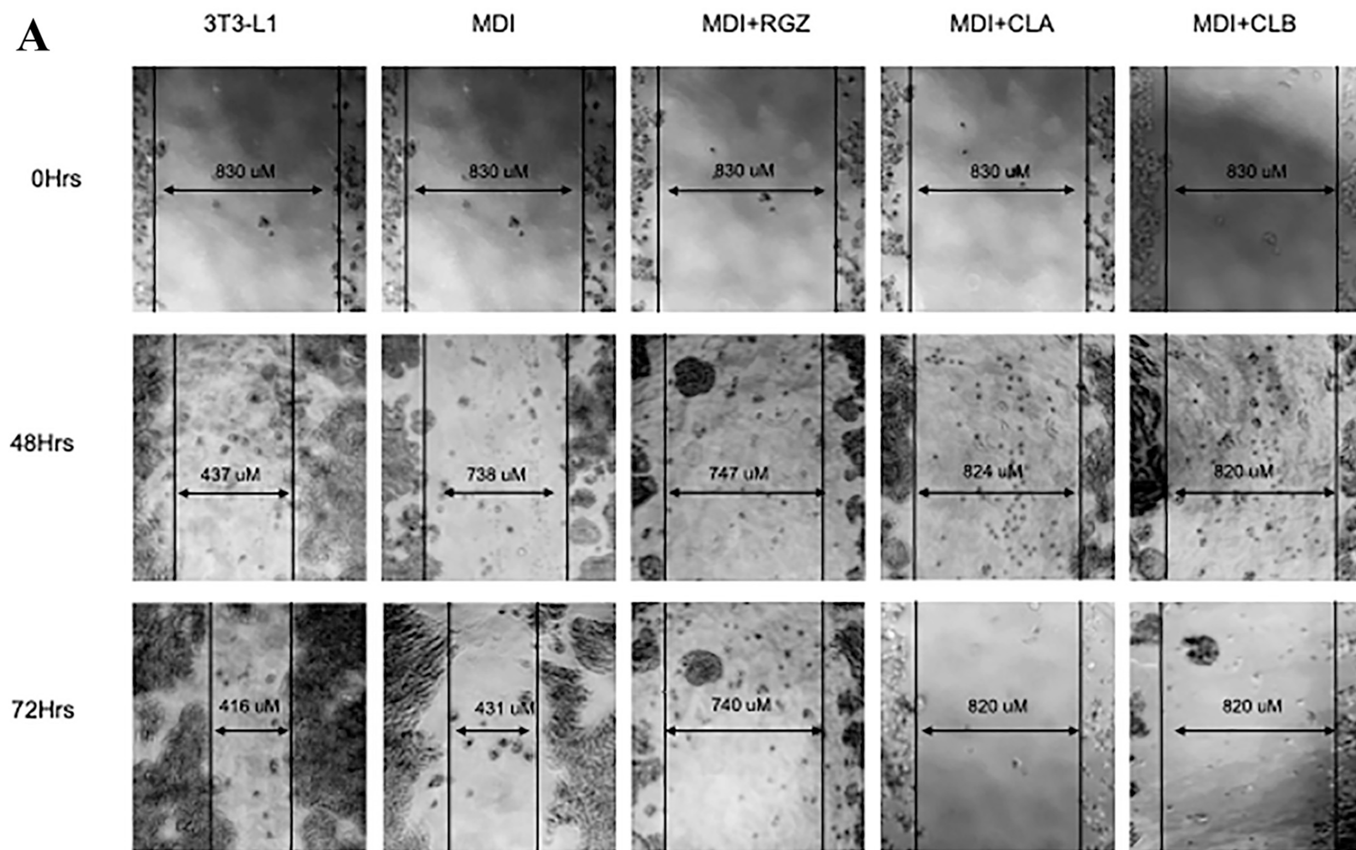
Fig. 6. Conditioned medium from 3T3-L1 cells treated with cladosporol A or cladosporal B inhibited HT-29 proliferation. 3T3-L1 cells were differentiated as described in section 2.1, and the medium of cells (CM) treated with MDI, MDI plus 5 μM rosiglitazone (MDI + RGZ) or MDI plus 5 μM cladosporol A (MDI + CL A) or MDI plus 5 μM cladosporol B (MDI + CL B) was collected, used to culture HT-29 cells for 48 h (A) and 72 h (B) and finally counted. The data are presented as the mean ± standard deviation of two different experiments performed in triplicate.

reported in Fig. 5D-G, further proved that cladosporol action required its binding to PPARγ. These data are consistent with those from our previous papers in which we demonstrated that the antiproliferative and proapoptotic activities of cladosporols against CRC-derived cells are mediated by binding of both these drugs to PPARγ [22,23,26].

It is intriguing to compare the cladosporol-mediated effects with the effects of RGZ, a well-known full agonist of PPARγ belonging to the TZD family. While RGZ strongly activated PPARγ, thus inducing differentiation of 3T3-L1 preadipocytes to mature adipocytes, both cladosporols, on the contrary, displayed a robust inhibition of PPARγ activity and an evident ability to arrest the differentiation process in the early phase (Fig. 2). RGZ has been identified as a full agonist of PPARγ, and in this role it is able to enter the PPARγ LBD pocket, where it binds to the specific functional group of some exposed amino acids. Specifically, RGZ forms canonical hydrogen bonds with residues Y473, H449 and H327 on the inner surface of H12, and stabilises the region, including loop 11/12, thus contributing to establish the network of contacts required for the activation of H12 that, in turn, allows recruitment of coactivators [35]. The data from our previous studies demonstrated that cladosporol A binds to the PPARγ pocket differently from RGZ, but it still behaves like a full agonist. In addition, we found that cladosporol B binds to the LBD pocket even more differently [26]. Interestingly, cladosporol B makes no direct contacts with H12, a hallmark of traditional TZDs, but preferentially stabilises H3 through closer hydrophobic contacts or hydrogen bonds with residues of this helix (S289, F282, Q283 and Q286). These contacts likely affect the recruitment of specific cofactors and generate a different transactivation potential [26]. On the basis of the experiments carried out in these previous studies, we affirmed that cladosporol A works as a peculiar PPARγ full agonist, whereas cladosporol B is a proper partial agonist of PPARγ [36]. On the strength and specificity of these diverse modes of binding of cladosporol A and cladosporol B, we suggest that both these drugs specifically induce, in the milieu of 3T3-L1 cells committed to differentiate to mature adipocytes, changes in the PPARγ LBD structure that cause the recruitment of different cofactors. Thus, cladosporol A and cladosporol B might inhibit particular differentiation pathways in 3T3-L1 cells and ultimately mediate the antiadipogenic function.

Adipose tissue is an endocrine organ that can synthesise and secrete several adipocytokines that are involved in the control of energy homeostasis [37]. An increase in adipose tissue results in adipocytokine dysregulation that, in turn, causes serious metabolic diseases [38]. Adiponectin, an adipocytokine, is usually synthesised in mature adipocytes and secreted in the blood to stimulate insulin action [39]. The plasma adiponectin level in obese animal models and human patients is very low; this phenomenon indicates that a reduced concentration of adiponectin might cause development of obesity-dependent metabolic complications such as type 2 diabetes [40,41]. Diminished plasma adiponectin levels during tumour development in different tissues have been observed [42–44]. Moreover, various studies have demonstrated there is a close correlation between low levels of plasma adiponectin and the risk of colon cancer [45,46]. Finally, it is well known that adiponectin exerts antiproliferative and proapoptotic activities on CRC-derived cells [47]. Adipocytes regulate the tumour microenvironment by secreting adipokines to modify cancer promotion and progression. Hence, we wondered whether factors secreted by undifferentiated and differentiated 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B could influence the growth and migration of HT-29 cells. There was evident inhibition of HT-29 proliferation and migration when these cells were cultured with conditioned medium from differentiated 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B compared with the controls (undifferentiated preadipocytes and differentiated 3T3-L1 cells treated with MDI plus RGZ) (Figs. 6 and 7). These data suggest a cladosporol-treatment-dependent break in the crosstalk between 3T3-L1 mature adipocytes and HT-29 cells. Inhibition of growth and migration of HT-29 cells appears to be induced by increased adiponectin synthesis and secretion in the medium from differentiated 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B compared with the controls (undifferentiated preadipocytes and differentiated 3T3-L1 cells treated with MDI plus RGZ) (Fig. 8A and B). The elevated adiponectin protein level in the conditioned medium from differentiated 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B was also accompanied by diminished leptin synthesis and secretion (Fig. 8A and B). It is well known that leptin works as a mitogenic and antiapoptotic factor towards CRC-derived cells, whereas adiponectin displays robust antiproliferative and proapoptotic activities in the same kind of cells [48–50]. We demonstrated that cladosporols can differentially modulate adiponectin and leptin expression and their secretion in the medium, thus suggesting that both drugs could interfere with the relative pathways induced by these adipocytokines. It is reasonable that the simultaneous decrease of leptin and the increase of adiponectin in the medium, collected from differentiated 3T3-L1 cells treated with MDI plus cladosporol A or cladosporol B, may mediate the inhibitory effects on HT-29 cell proliferation and migration (Fig. 7D).

In conclusion, our findings confirm the role of cladosporols as specific inhibitors of mature adipocyte differentiation. Both cladosporol A and cladosporol B inhibited the expression of adipogenesis-related



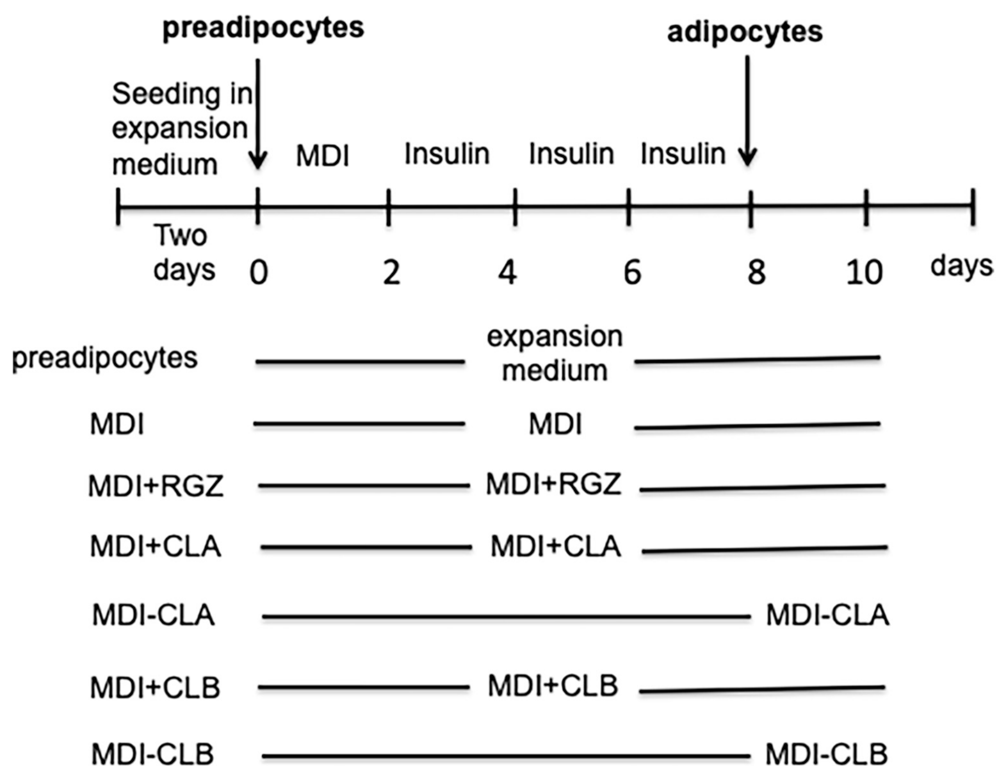
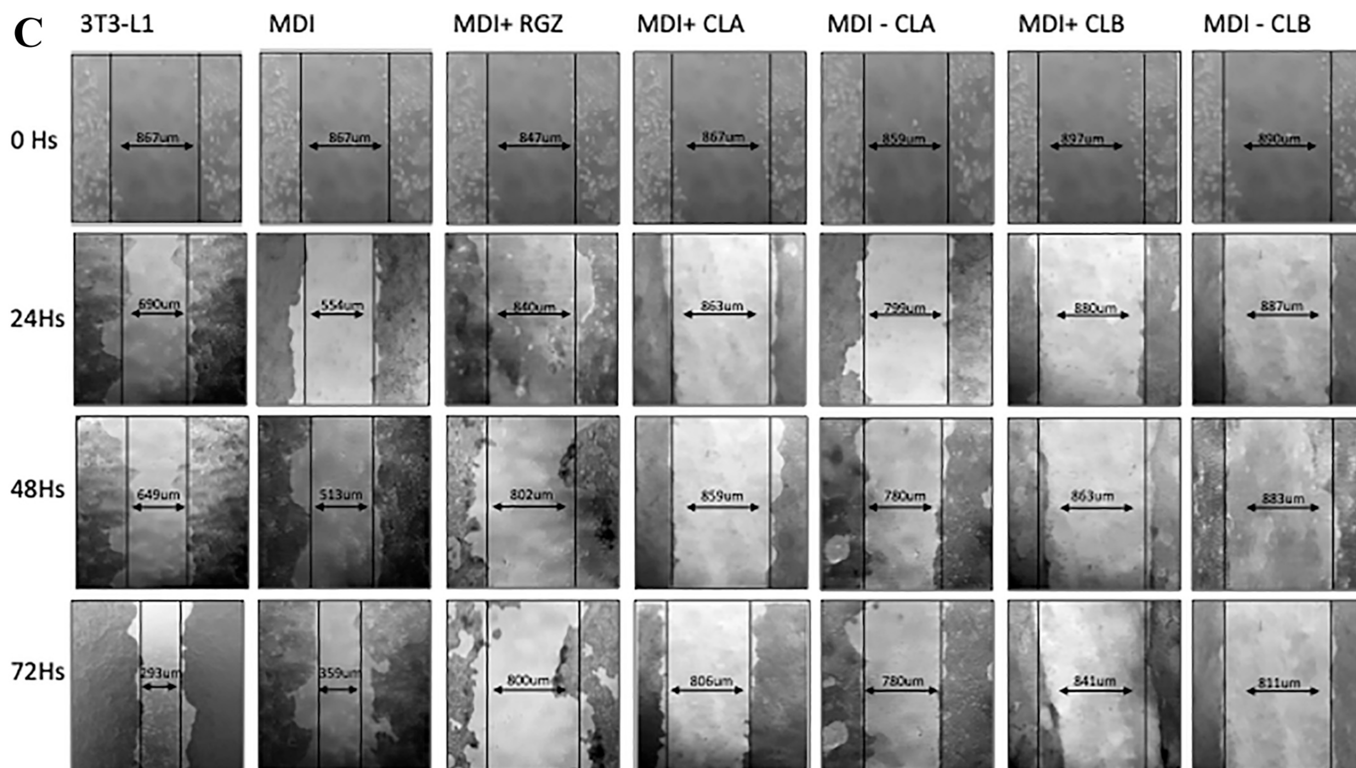


Fig. 7. Conditioned medium from 3T3-L1 cells treated with cladosporol A or cladosporol B inhibited HT-29 migration and invasion. (A) 3T3-L1 preadipocytes were differentiated as described in section 2.1, and the medium from cells treated with MDI, MDI plus 5 μM rosiglitazone (MDI + RGZ) or MDI plus 5 μM cladosporol A

(MDI + CL A) or MDI plus 5 μM cladosporol B (MDI + CL B) was collected. HT-29 cells were plated at a subconfluent density (8.0×10^4 cells/ml) in growth medium. Once they reached 100% confluence, a wound was formed using the plastic micropipette tip. Conditioned medium was used to culture HT-29 cells for 48 and 72 h. (B) The scheme indicates variations in the 3T3-L1 preadipocyte differentiation and cladosporol treatment protocols used in the migration assay shown in (C). (C) The assay was carried out as described in (A), except that the conditioned medium was used to culture HT-29 cells for 24, 48 and 72 h and the medium collected from 3T3-L1 cells treated with MDI plus CL A and CL B was deprived of both drugs in the last two days of cell differentiation (the new samples are indicated as MDI-CL A and MDI-CL B). (D) Western blotting analysis of cell cycle markers and apoptotic factors in HT-29 cells treated as described in (C). Total protein extracts from HT-29 cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nylon membranes. Subsequently, antibodies were used to detect the expression of β-catenin, cyclin D1, p21 and caspase 3. To control the samples loaded derived from untreated and cladosporol A- and cladosporol B-treated HT-29 cells and normalize the results, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of protein/β-actin of at least 2 independent experiments.



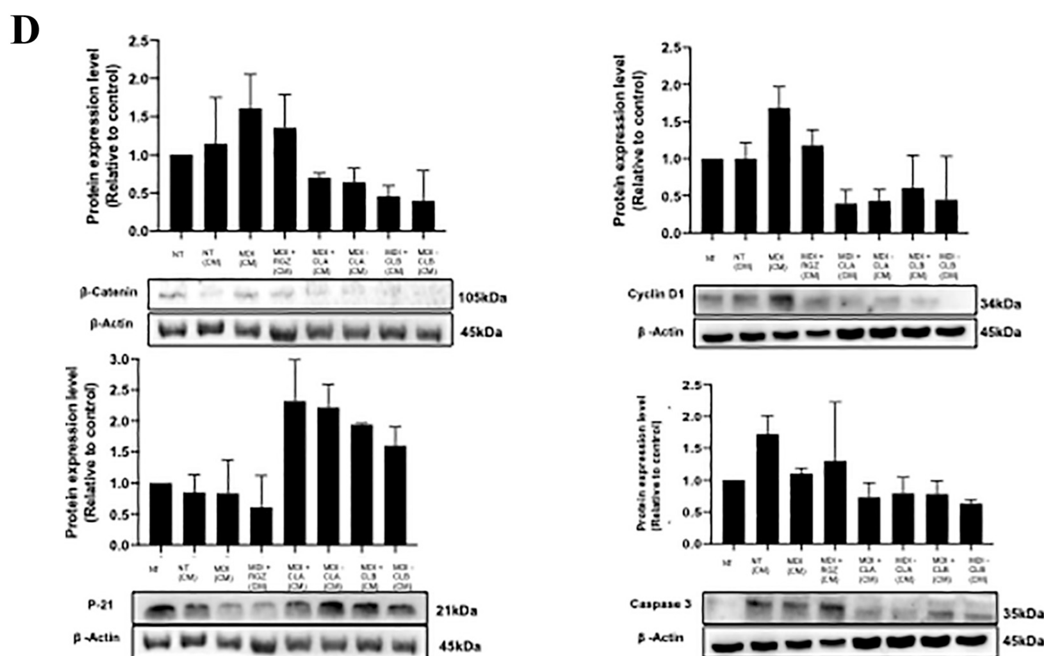


Fig. 7. (*continued*).

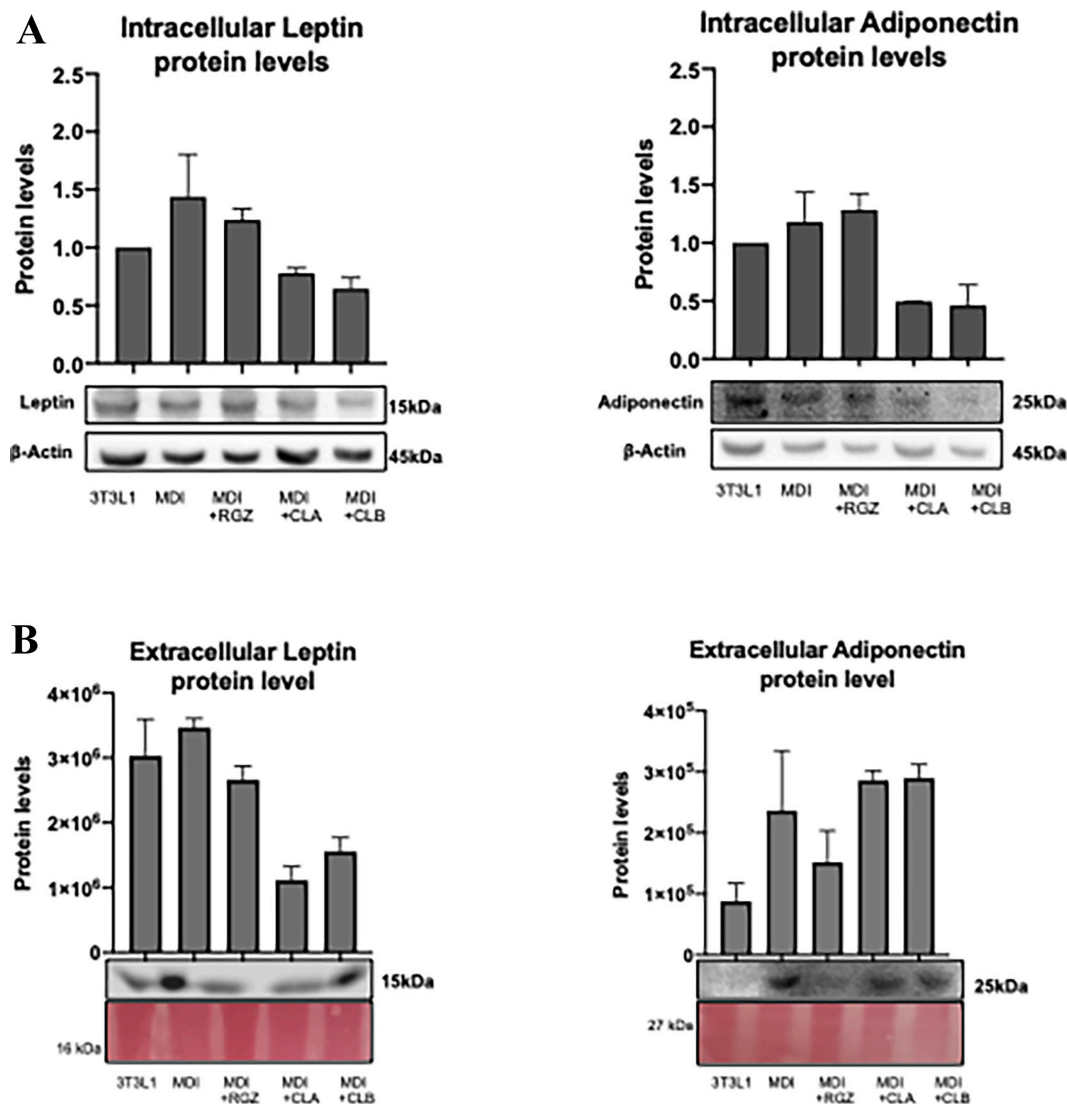


Fig. 8. Cladosporol A and cladosporol B inversely regulated the synthesis and secretion of leptin and adiponectin in mature 3T3-L1 adipocytes. 3T3-L1 preadipocytes were differentiated as described in section 2.1, and the medium from cells treated with differentiation mix MDI, MDI plus 5 μM rosiglitazone (MDI + RGZ) or MDI plus 5 μM cladosporol A (MDI + CL A) or MDI plus 5 μM cladosporol B (MDI + CL B) was collected. Cellular and medium proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoreis and transferred to nylon membranes. (A) Western blotting analysis of cellular leptin and adiponectin protein levels from 3T3-L1 cells. To control the samples loaded derived from untreated and cladosporol A- and cladosporol B-treated 3T3-L1 cells and normalize the results, an anti-β-actin antibody was used. The bar graphs represent the mean ± SD of intracellular protein/β-actin of at least 2 independent experiments. (B) Western blotting analysis of secreted leptin and adiponectin protein levels from 3T3-L1 cells. To control the samples loaded derived from untreated and cladosporol A- and cladosporol B-treated 3T3-L1 cells and normalize the results, a comparison with the protein samples was used after the Ponceau staining. The bar graphs represent the mean ± SD of specific protein/red band of Ponceau of at least two independent experiments. The molecular weights of 16 and 27 kDa indicate the position of leptin and adiponectin in the gel.

proteins, including PPARγ and c/EBPα. In addition, cladosporol A and cladosporol B inversely regulated the synthesis and secretion of leptin and adiponectin; this action influences the proliferation and migration of HT-29 culture cells. Indeed, cladosporols display the ability to break the crosstalk between mature adipocytes and CRC cells. This action offers a promising approach to inhibit concomitantly adipogenesis and the progression of CRC cells and tumors.

CRediT authorship contribution statement

Roberta Rapuano: Investigation, Methodology, Formal Analysis, Writing–Original Draft, Writing–Review & Editing. **Pamela Ziccardi**: Investigation, Methodology, Formal Analysis. **Valentina Cioffi**: Investigation. **Sabrina Dallavalle**: Methodology. **Salvatore Moricca**: Methodology. **Angelo Lupo**: Methodology, Formal Analysis, Supervision, Writing–Original Draft, Writing–Review & Editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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