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The antiproliferative and proapoptotic effects of cladosporols A and B are related to their different binding mode as PPARγ ligands

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ABSTRACT

Cladosporols are secondary metabolites from *Cladosporium tenuissimum* characterized for their ability to control cell proliferation. We previously showed that cladosporol A inhibits proliferation of human colon cancer cells through a PPARγ-mediated modulation of gene expression. In this work, we investigated cladosporol B, an oxidate form of cladosporol A, and demonstrate that it is more efficient in inhibiting HT-29 cell proliferation due to a robust G0/G1-phase arrest and $p21^{waf1/cip1}$ overexpression. Cladosporol B acts as a PPARγ partial agonist with lower affinity and reduced transactivation potential in transient transfections as compared to the full agonists cladosporol A and rosiglitazone. Site-specific PPARγ mutants and surface plasmon resonance (SPR) experiments confirm these conclusions. Cladosporol B in addition displays a sustained proapoptotic activity also validated by $p21^{waf1/cip1}$ expression analysis in the presence of the selective PPARγ inhibitor GW9662. In the DMSO/H_2O system, cladosporols A and B are unstable and convert to the ring-opened compounds **2A** and **2B**. Finally, docking experiments provide the structural basis for full and partial PPARγ agonism of **2A** and **2B**, respectively. In summary, we report here, for the first time, the structural characteristics of the binding of cladosporols, two natural molecules, to PPARγ. The binding of compound **2B** is endowed with a lower transactivation potential, higher antiproliferative and proapoptotic activity than the two full agonists as compound **2A** and rosiglitazone (RGZ).

1. Introduction

Natural molecules isolated and characterized from plants, fungi and microorganisms represent a source of new drugs for cancer treatment. Their employment is advantageous because they generally are less expensive and act as multitargeted therapeutics, a feature that is absolutely beneficial to the treatment of solid tumors caused by dysregulation of several genes. Natural compounds usually have lower affinity and thus lower toxicity than synthetic drugs so that they do not interfere with most of the essential biological functions.

Among natural compounds, carotenoids, flavonoids, organosulfurs, isothiocyanates, indoles and monoterpenes have been associated with a reduced risk of cancer in a large series of epidemiological and preclinical studies [1–3]. In addition, they are very useful in the management of cancer patients either alone or in combination with known molecules [4–6].

In the search for novel natural molecules, we recently demonstrated that cladosporol A, a secondary metabolite from *Cladosporium tenuissimum*, exhibits antiproliferative properties in a variety

Abbreviations: CAD, caspase-activated deoxyribonuclease; CRC, colorectal cancer; HR-ESI-MS, high resolution electrospray ionization mass; LBD, ligand-binding domain; MDA, malondialdehyde; NCoEx, nuclear corepressor exchange factor; PPARs, peroxisome proliferator-activated receptors; PBS, phosphate saline buffer; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, PPAR response element; POX/PRODH, proline oxidase/proline dehydrogenase; RGZ, rosiglitazone; RSV, Rous Sarcoma Virus; RXR, retinoid X receptor; ROS, reactive oxygen species; SPPARMs, Selective PPARγ Modulators; SPR, surface plasmon resonance; TCF, T-cell factor; TMS, tetramethylsilane; TZDs, thiazolinediones.

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of human colon cancer cell lines through modulation of several cell cycle gatekeeper gene expression ($p21^{waf1/cip1}$, cyclin D1, cyclin E, CDK2, CDK4) [7]. These effects are mediated by PPARγ to which Cladosporol A binds as a ligand, activating, in turn, $p21^{waf1/cip1}$ gene expression in an Sp1-dependent manner [8]. Cladosporol A-bound PPARγ targets β-catenin to proteasomal degradation, reducing the overall amount of the protein and transcription of its target genes [8]. In addition, it stimulates E-cadherin gene transcription, further supporting the anti-metastatic activity of this compound [8]. These results are consistent with the data from literature disclosing for PPARγ a protective role in *in vitro* and *in vivo* colorectal cancer (CRC) models [9–13]. In the gastrointestinal tract, in fact, PPARγ impairs cellular proliferation, stimulates differentiation and induces apoptosis [14]. Both in sporadic CRCs and rodent models PPARγ hampers tumor initiation/progression acting as a tumor suppressor [15]. CRC derived cells and transplanted tumors in nude mice undergo growth inhibition, G0/G1 arrest and caspase-activated apoptosis and differentiation upon treatment with selective ligands, such as thiazolinediones (TZDs) or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 [16,17]. Moreover, ligand-bound PPARγ regulates lipid and carbohydrate metabolism, adipogenesis, insulin sensitization, inflammation, atherosclerosis [18–20]. It is conceivable that such a wide range of functions implies different binding modalities of the selective ligands to the receptor ligand binding domain (LBD) and interactions with protein partners activating different pathways involved in the control of metabolism, proliferation or differentiation. Such a choice likely depends on the amount of the receptor available in a given cell, the chemical features of the ligands and their affinity for the receptor. The ligands, moreover, induce conformational changes to the PPARγ LBD that influence the recruitment of functional effectors (coactivators, corepressors, molecular adapters, chromatin modifying enzyme activities etc.) that in turn stimulate the formation of an open chromatin and activation of a wide range of gene transcription. Many of these genes are involved in glucose and lipid metabolism, thus TZD-activated PPARγ ameliorates diabetic patients' conditions [21–24]. Unfortunately, these synthetic TZDs cause undesired side- and off-target effects (higher rate of bone fractures, weight gain, edema, renal function failure, etc.) [25–27]. More recently, identification and characterization of specific PPARγ ligands known as SPPARMs (Selective PPARγ Modulators), displaying beneficial antidiabetic action with no or reduced side-effects, are gaining interest [28–30].

In line with this reasoning, to obtain novel molecules that could act as PPARγ agonists and inhibit CRC development, we selected cladosporol B, an oxidate form of cladosporol A, and investigated its antiproliferative and proapoptotic properties in comparison with cladosporol A (Fig. 1). Here, we demonstrate that cladosporol B displays anticancer activity in HT-29 cells due to not only a G0/G1 cell cycle arrest via $p21^{waf1/cip1}$ early overexpression, but also a strong activation of apoptosis as evidenced by experiments in different cell lines in the presence of GW9662, a specific PPARγ inhibitor. The effects of cladosporol B are more pronounced than those of cladosporol A and correlate with a lower affinity for the PPARγ LBD and a reduced PPRE-mediated transactivation potential. Surface plasmon resonance (SPR) experiments and site-specific PPARγ mutants confirmed these conclusions. Proton nuclear magnetic resonance (^{1}H NMR) and high resolution electrospray ionization mass (HR-ESI-MS) were used to elucidate the epoxide ring opening reaction of cladosporols A and B in dimethyl sulfoxide (DMSO)/H_2O system to form the corresponding PPARγ active compounds **2A** and **2B**. Moreover, we report and compare the 3D structures of the PPARγ LBD in the complex with **2A** and **2B**, providing a molecular explanation for their different behavior as full and partial PPARγ agonists, respectively. The stronger antiproliferative activity of cladosporol B might correlate with a differential binding to the PPARγ LBD as compared to cladosporol A.

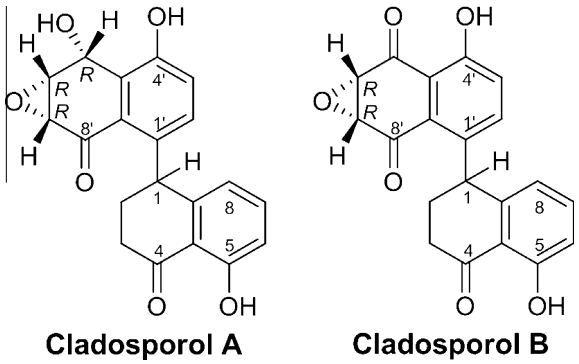

Fig. 1. Chemical structures of cladosporol A and B.

2. Materials and methods

2.1. Cells, antibodies and reagents

HT-29, RKO and HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). HT-29 cells bear different genetic abnormalities typical of human CRC, such as a mutated *Tp53* (Arg 273 His) and a wild-type *RAS* allele [31].

Antibodies against $p21^{waf1/cip1}$, β-actin and caspase 3 precursor were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse and anti-rabbit IgG peroxidase-linked secondary antibodies, ECL and ECL Plus Western blotting detection kit were purchased from Amersham Life Science (Little Chalfont, Buckinghamshire, UK). D-MEM (Dulbecco's Modified Eagle's Medium), D-luciferin sodium salt, trichloroacetic acid, propidium iodide (PI), rosiglitazone (RGZ) and GW9662 were from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin–streptomycin, L-glutamine, trypsin–EDTA and OptiMEM I were obtained from Gibco (Carlsbad, CA, USA). Charcoal/dextran-treated FBS was purchased from Hyclone (Logan, Utah, USA). Fetal calf serum (FCS), Lipofectamine 2000, CellTiter Aqueous One Cell Proliferation Assay were obtained from Promega (Madison, WI, USA).

2.2. Cell culture and cladosporol A and B treatments

Human colon adenocarcinoma HT-29 or RKO cells were grown as a monolayer in D-MEM containing 10% FBS, 1% penicillin–streptomycin and 1% L-glutamine. The cells were cultured in 100 mm plates, at 70–80% confluence, in a 5% CO_2 humidified atmosphere, at 37 °C. Cladosporol A and B (Fig. 1) treatments were carried out in the presence of 10% charcoal/dextran-treated FBS containing 1% penicillin–streptomycin and 1% L-glutamine, whereas the cells were maintained at 50% confluence. Cladosporol A and B were dissolved in 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%. In separate experiments, cells were treated with increasing concentrations of cladosporol A and B, for the indicated times.

2.3. Cell viability

The growth rate of HT-29 cells was evaluated using the CellTiter Aqueous One Cell Proliferation Assay (Promega, Madison, WI, USA), as previously described [7]. To further evaluate cell growth after treatment with cladosporol A and B, cells were plated in 12-well plates at a density of 10^6 cells/cm^2. After treatment, the cells were washed with PBS, trypsinized and collected in culture medium. Cell counting was performed as previously reported [7].