Biochemical and Biophysical Research Communications 450 (2014) 652–658

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anti-proliferative and pro-apoptotic activity of whole extract and isolated indicaxanthin from *Opuntia ficus-indica* associated with re-activation of the onco-suppressor p16^{INK4a} gene in human colorectal carcinoma (Caco-2) cells

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ARTICLE INFO

Article history:
Received 3 June 2014
Available online 14 June 2014

Keywords:
Indicaxanthin
Colorectal carcinoma
In vitro
Epigenetic control
Cell cycle

ABSTRACT

Phytochemicals may exert chemo-preventive effects on cells of the gastro-intestinal tract by modulating epigenome-regulated gene expression. The effect of the aqueous extract from the edible fruit of *Opuntia ficus-indica* (OFI extract), and of its betalain pigment indicaxanthin (Ind), on proliferation of human colon cancer Caco-2 cells has been investigated. Whole extract and Ind caused a dose-dependent apoptosis of proliferating cells at nutritionally relevant amounts, with IC_{50} 400 ± 25 mg fresh pulp equivalents/mL, and 115 ± 15 μM (n = 9), respectively, without toxicity for post-confluent differentiated cells. Ind accounted for ~80% of the effect of the whole extract. Ind did not cause oxidative stress in proliferating Caco-2 cells. Epigenomic activity of Ind was evident as de-methylation of the tumor suppressor *p16*^{INK4a} gene promoter, reactivation of the silenced mRNA expression and accumulation of p16^{INK4a}, a major controller of cell cycle. As a consequence, decrease of hyper-phosphorylated, in favor of the hypo-phosphorylated retinoblastoma was observed, with unaltered level of the cycline-dependent kinase CDK4. Cell cycle showed arrest in the G2/M-phase. Dietary cactus pear fruit and Ind may have chemo-preventive potential in intestinal cells.

1. Introduction

Vegetables and phytochemicals have important roles in reducing the risk of various chronic and degenerative diseases [1]. While being a major cause of death in the Western world, colorectal cancer has appeared less frequent among people consuming traditional dietary regimens rich of fruit and vegetables [2]. Cactus pear is popular and fruits are largely consumed in the Mediterranean basin. Long-established, the beneficial properties of this plant have been object of sound scientific work in the latest two decades. In these studies betalain components emerged as very significant phytochemicals with the potential to maintain human well being [3]. Betalains include two classes of pigments, red betacyanins and yellow betaxanthins [4]. Antitumoral potential of betalain-rich extracts from dietary sources has been explored in animal models and in cancerous cell lines [5–7]. In addition betanin, the main dietary betacyanin, has been shown to inhibit the growth of breast, colon, stomach, CNS, and lung cancer cells [8], induce apoptosis in K562 human myeloid leukemia cell lines [9], and have a weak activity on the epigenome-regulated gene expression of MCF-7 breast [10], but not HepG2 liver [11] cancer cells. Potential anti-proliferative and/or chemo-preventive and epigenetic activity of betaxanthins have not been explored so far.

Indicaxanthin (Ind) is highly concentrated in the edible fruits of the Sicilian cactus *Opuntia ficus-indica*. We showed that this betaxanthin behaves as a radical scavenger and antioxidant [12–14], interacts with and locates in membranes [15,16], and affects cell redox-sensitive signaling pathways [17,18]. When consuming cactus pear fruits, Ind is stable at the digestive conditions, not metabolized by the enterocytes, and highly bioavailable in humans in its native form [19–21]. Remarkable anti-inflammatory activity and effects have recently been shown in differentiated intestinal epithelial cells exposed to the action of the pro-inflammatory interleukin IL-1β [22], and in rats after oral administration of dietary-consistent amounts [23].

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http://dx.doi.org/10.1016/j.bbrc.2014.06.029
0006-291X/

The progression of colorectal cancer from normal colonic epithelium to the acquisition of the malignant phenotype is accompanied by numerous genetic and epigenetic alterations, which highlights the importance of dietary compounds as eventual chemo-preventive factors. This study first investigated the antiproliferative activity of a fruit extract from *O. ficus-indica* (OFI extract), and of purified Ind, on colon adeno-carcinoma (Caco-2) cells, then explored the Ind potential to revert epigenetic alterations and re-activate the expression of $p16^{INK4a}$, a onco-suppressor gene abnormally methylated in colon cancer.

2. Materials and methods

Unless stated, all materials were from Sigma Chemical Co. (St. Louis, MO, USA) and solvents of the highest purity.

2.1. OFI extract and Ind preparation

Aqueous extract from *O. ficus-indica* fruits (yellow cultivar) was prepared as described [12], and Ind isolated from the extract and quantified as reported [12,24].

2.2. Cell culture and treatment

Caco-2 cells (American Type Culture Collection Rockville, MD), were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 2 mmol/L L-glutamine, 1% non-essential amino acids, 10 mM HEPES, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin and were maintained at 37 °C in 5% CO_2, and 95% humidity, with changing medium 2–3 times per week. Mycoplasma contamination was monitored by phase contrast microscopy and fluorescent staining of DNA.

Cell sensitivity to OFI extract or Ind was evaluated by MTT assay [25].

2.3. Measurement of apoptosis

FACS determination of phosphatidylserine (PS) exposure was by double staining of cells with Annexin V/propidium iodide (PI), in accordance with the Annexin V apoptosis detection kit FITC (Cat. No. 88-8005, eBiosciences Inc., San Diego, CA, USA); Epics XL™ flow cytometer with Expo32 software (Beckman Coulter, Fullerton, CA).

Mitochondrial trans-membrane potential ($\Delta\psi_m$) was assayed by FACS, using 3,3′-dihexyloxa-carbocyanine iodide (DiOC6(3), Molecular Probes Inc., Eugene, OR, USA), a cationic dye accumulating in the mitochondrial matrix. Membrane potential changes are indicated by a reduction in the DiOC6-induced fluorescence intensity.

2.4. Analysis of cell cycle

Cell cycle was analyzed by FACS. For synchronization, cells were cultured in serum-free medium for 24 h. After addition of Ind, cells were re-stimulated with 10% FBS and incubation prolonged for the required times. Then cells were harvested by trypsinization and washed with PBS. Cells (5.0×10^5) were incubated in the dark in 0.5 mL PBS containing 20 μg/ml PI and 200 μg/ml RNase, for 30 min at room temperature.

2.5. Measurement of reactive oxygen species (ROS) and thiols

ROS level was evaluated by changes of fluorescence resulting from intracellular oxidation of 10 μM (final concentration) dichloro-dihydro-fluorescein diacetate (DCFDA), added to the cell medium 30 min before the end of treatment. After trypsinization cells were centrifuged, collected, washed, and suspended in PBS followed by FACS analysis. Thiols were measured in cells washed twice with ice-cold PBS containing 0.025% butylated hydroxytoluene and sonicated. Cell lysates were mixed with 10% SDS and 30 μM 5,5′-dithiobis-(2-nitrobenzoic acid), and incubated with shaking at room temperature for 30 min. Thiols (protein-SH and glutathione) were measured spectrophotometrically at 412 nm.

2.6. Methylation-sensitive restriction endonucleases multiplex-polymerase chain reaction (MSREM-PCR)

DNA was extracted and used in MSREM-PCR to analyze the CpG island of $p16^{INK4a}$ gene promoter region as described [26] with some modifications. DNA (0.25 μg) was digested with excess of *CfoI* methylation-sensitive restriction endonuclease (MSRE) and amplified by multiplex-PCR in the presence of oligos flanking sites located within the CpG island of $p16^{INK4a}$ gene promoter (426 bp product; forward: 5′-ACTCCCTCCCCATTTTCCTAT CT-3′ and reverse: 5′-CCGCGATACAAC CTTCCTAACT-3′). Co-amplification of IL-4 internal region was concurrently carried out as a control (998 bp product; forward: 5′-CCCCAAGTGACT GACAATCTGG-3′ and reverse: 5′-GTGAGAGTATTTGG TTTTTCAGAAAT-3′). The MSREM-PCR products were resolved as described [27].

2.7. Real-time polymerase chain reaction (RT-PCR) of $p16^{INK4a}$

Total RNA was extracted using Trizol (Invitrogen) according to manufacturer's protocols; the RNA was eluted in diethyl pyrocarbonate (DEPC) treated water (0.01% DEPC) and stored at −80° until RT-PCR analysis. Nucleic acid concentrations were measured by spectrophotometry (NanoDrop 1000 Spectrophotometer). First-strand cDNA was synthesized from total RNA (250 ng) using a SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. mRNA expression was determined using first strand cDNA as the template by quantitative real-time PCR (qPCR), with Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen) in 7300 Real Time PCR system (Applied Biosystem, Philadelphia, PA, USA). The sequences of the $p16^{INK4a}$ primers used were: sense (5′-GTGGACCTGGCTGAGGAG-3′) and antisense (5′-CTTTCAA TCGGGGATGTCTG-3′). β-Actin was the internal control with sense (5′-GCCCACAT AGGAATCCTTCTGAC-3′) and antisense (5′-AGGCACCAGGGCGT GAT-3′) primers (283-bp product). PCR data were analyzed by the Relative Quantification Study Software (Applied Biosystem) and expressed as target/reference ratio. In our approach calibrator normalized relative quantification corrected for PCR efficiency.

2.8. Western blot analysis

Cells were rinsed twice with ice-cold PBS, harvested by scraping with hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 1.5 μg/ml soya bean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine and 0.5 mM DTT) and incubated 15 min on ice. Lysates were centrifuged (13,000*g*, 5 min) and cytosolic fraction stored at −80 °C up to two weeks. 30 μg protein (Bradford assay reagent, Bio-RAD, Milan, Italy) was separated by 8–12% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Immunoblots were incubated overnight at 4 °C with blocking solution (5% skim milk), followed by incubation with anti-$p16^{INK4a}$ monoclonal antibody (clone F-12, Cat No sc-1661, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-CDK4 monoclonal antibody (clone C-22, Cat No sc-260 Santa Cruz Biotechnology), or anti-retinoblastoma protein (RB, clone G3–245, Cat No 554136, BD Biosciences, San Jose,

CA, USA), 1 h, room temperature. Blots were washed two times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated anti-IgG antibody, 1 h, room temperature. Blots were washed five times with TTBS and developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA). Immunoreactions were also performed using β-tubulin (clone H-235, Cat no sc-9104, Santa Cruz Biotechnologies) antibody as loading control. Immunoblots were submitted to densitometric scanning and analyzed by SigmaGel Image Analysis software reported above.

2.9. Statistical analysis

Calculations and graphs were by INSTAT-3 statistical software (GraphPad software Inc, San Diego, CA, USA). Data are means ± SD of three triplicate observations. Comparison between individual group means was performed by unpaired Student's *t*-test. One-way ANOVA with Bonferroni's post hoc test was used for multiple comparisons. Significance was accepted if the null hypothesis was rejected at $P < 0.05$ level.

3. Results and discussion

3.1. Pro-apoptotic effects of OFI extract and Ind

Treating proliferating Caco-2 cells with either OFI extract, or the equivalent amounts of purified Ind, for 48 h, caused a dose-dependent growth inhibition (Fig. 1), with IC_{50} 400 ± 25 mg fresh pulp equivalents/mL and 115 ± 15 μM ($n = 9$), for extract and Ind, respectively. Neither the extract nor Ind affected the viability of Caco-2 cells grown 15 days post-confluence, a condition allowing their spontaneous differentiation, indicating absence of toxicity for non-malignant cells (Fig. 1a).

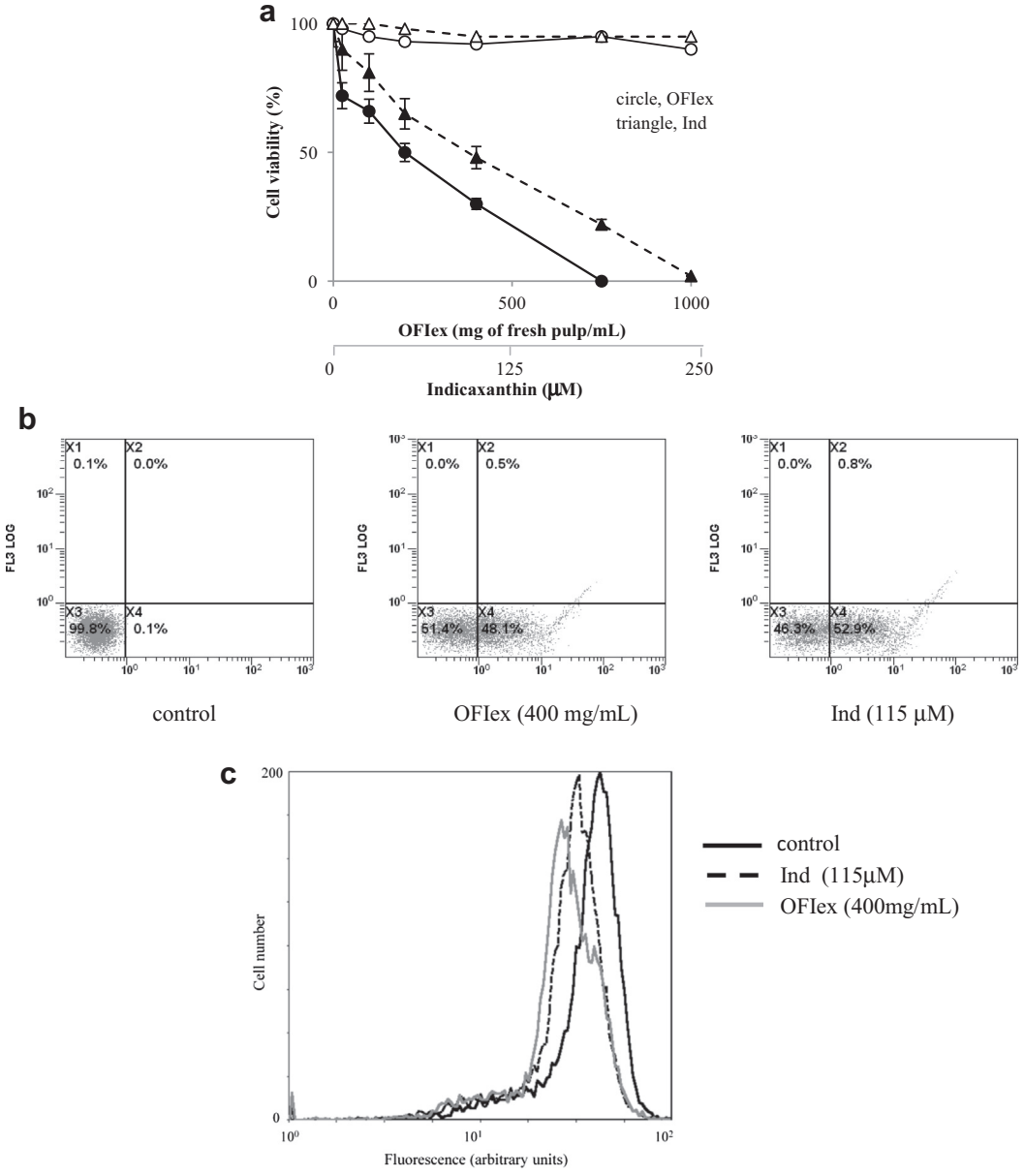

Fig. 1. Anti-proliferative (A) and pro-apoptotic (B) and (C) effects of OFI extract and Ind on Caco-2 cells after 48 h incubation. (A) viability of proliferating (closed symbol) or differentiated (open symbol) Caco-2 cells. Values are means ± SD of three triplicate experiments. (B) PS exposure (% of Annexin V/PI double stained cells) and (C) mitochondrial dysfunction (fluorescence intensity of DiOC6-treated cells) in Caco-2 cells incubated in the absence (control) or in the presence of OFI extract. Images representative of three triplicate experiments.

PS externalization evaluated by flow cytometry of annexin V-FITC-positive and PI stained Caco-2 cells, after 48 h treatment with either OFI extract (400 mg fresh pulp/mL) or Ind (115 μM), showed cells in early apoptosis (Fig. 1b). The absence of Annexin V-FITC$^-$/PI$^+$ cells ruled out necrosis. Contribution of mitochondria to the apoptotic process was investigated by fluorescent mitochondria-specific and voltage-dependent DiOC6(3) dye. Treatment with either 400 mg fresh pulp/mL extract or 115 μM Ind for 48 h caused decrease of DiOC6(3) uptake, indicating dissipation of $\Delta\psi_m$ (Fig. 1c). On the whole our data showed that extracts from the yellow fruit of *O. ficus-indica* caused growth arrest of adeno-carcinoma Caco-2 cells, and suggested involvement of Ind. When comparing the anti-proliferative effectiveness of isolated Ind with that of the whole extract, the betalain would account for about 80% of the activity. Assessing to what extent other components may have affected its activity in the extract is not possible, however the effectiveness when alone suggested that Ind could have a prominent role. Further studies with the purified compound have then been performed to investigate mechanistic aspects and eventual activity of Ind in controlling events associated with cell division and growth.

3.2. Cellular and genomic activity of Ind

Generation of high levels of ROS and oxidative stress, with DNA damage and apoptosis, has been considered to explain anti-proliferative activity of phytochemicals under certain circumstances [28–32]. Ind is a redox-active compound [12], we then investigated whether its anti-proliferative activity was associated with oxidative unbalance. With respect to untreated cells, neither the level of intracellular ROS nor total thiols, as a measure of the global cell redox state, appeared significantly modified by 115 μM Ind within 6–48 h of incubation (Fig. 2), indicating that anti-proliferative effects and apoptosis did not depend on redox stress. Importantly, Ind did not exhibit toxicity on differentiated cells, at least at the assayed amounts, suggesting selective effects at the level of modified regulatory mechanisms in the transformed cells.

The discovery that phytochemicals may modulate the epigenome-regulated gene expression [33,34] has recently set new basis to understand their potential to prevent or revert malignant transformations. Alterations of DNA methylation pattern, with methylation at the 5′ position of cytosines of specific sequences rich in cytosine-guanine dinucleotides (CpG islands), is the best known epigenetic modification in mammalian DNA and is commonly found in human cancers [35]. In particular, methylation and silencing of CpG sequences in the promoter of *p16*^{INK4a}, a tumor suppressor gene implicated in the cell cycle control and apoptosis, has been reported in approximately 30% of colorectal tumors, and observed in the adeno-carcinoma Caco-2 cell line [36,37]. Effect of Ind on the methylation status of *p16*^{INK4a} was investigated. Caco-2 cells were treated with 10 μM and 50 μM Ind for 48 h, followed by MSRE-PCR analysis. Genomic DNA samples were then digested with the MSRE CfoI, followed by PCR amplification of *p16*^{INK4a} promoter using a gene-specific primer. Concomitant amplification of *IL-4* promoter was performed as a negative control. The de-methylating agent 5-azacytidine was used as a positive control. Treatment with 10 μM Ind did not provide evidence of loss of methylation, however a total disappearance of the methylation-specific *p16*^{INK4a} band was induced by 50 μM (Fig. 3a). Influence of Ind on the *p16*^{INK4a} gene expression was further researched by quantitative PCR and Western blot assays. Real-time PCR revealed that 48 h treatment of Caco-2 cells with 50 μM Ind resulted in a significant reactivation of the silenced mRNA expression and p16^{INK4a} protein accumulation (Fig. 3b and c). In addition, this

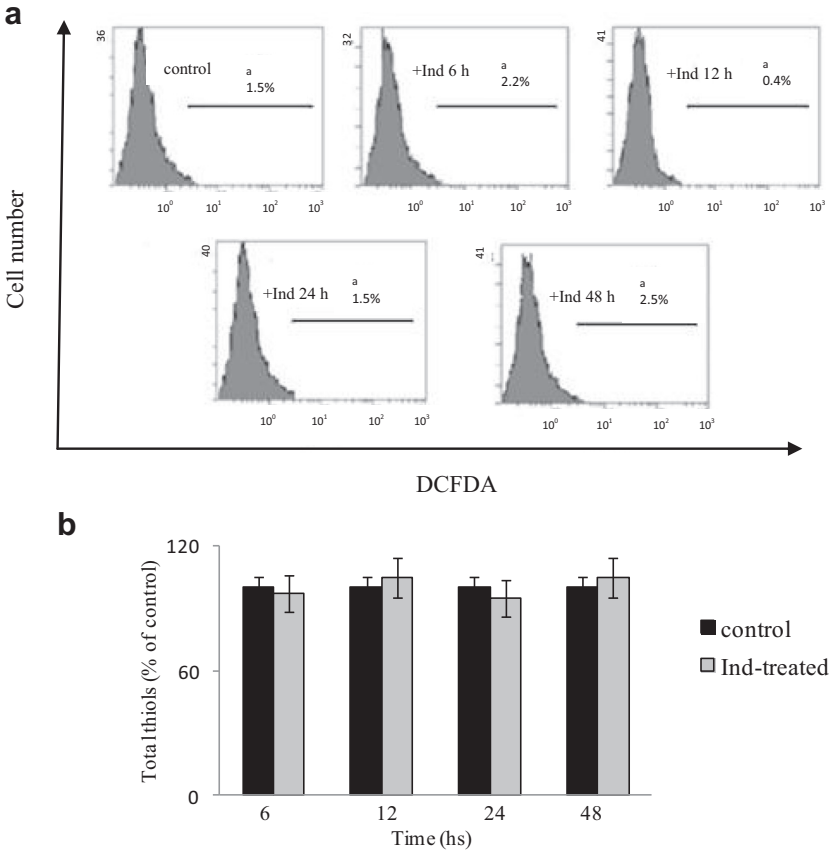

Fig. 2. Effect of Ind on (A) ROS and (B) total thiol production. Caco-2 cells were incubated either in the absence (control) or in the presence of 115 μM Ind for different time intervals. ROS were assayed by flow cytometry (DCFDA staining) and thiols by DTNB reaction. (A) Representative images and (B) means ± SD of three triplicate experiments.

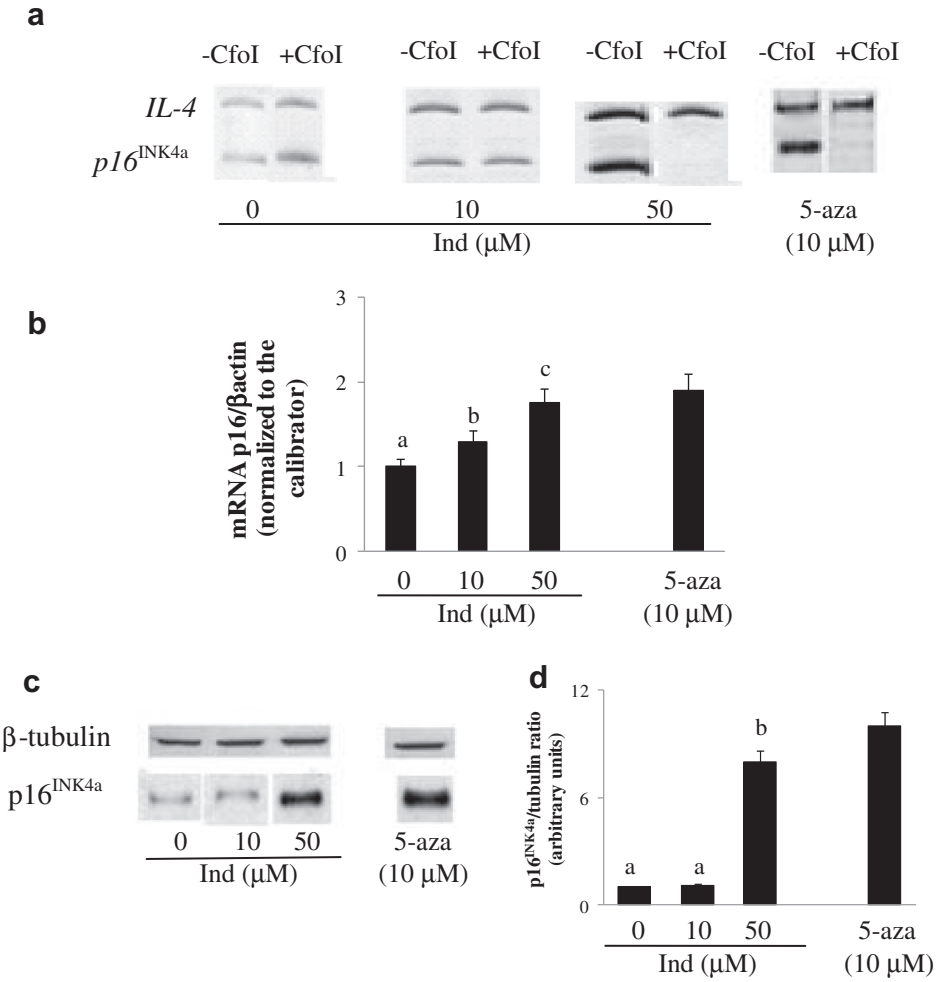

Fig. 3. Effect of Ind on reversal of methylation and reactivation of *p16*^{INK4a}. (A) Matched pairs of multiplex specific PCR products from undigested (-CfoI) or CfoI MSRE digested DNA (+CfoI) from either-untreated or Ind-treated Caco-2 cells for 48 h. *IL-4* gene promoter was used as negative control. (B) Real time-PCR of mRNA expression; β-actin, internal control. (C) Western blot and (D) densitometric analysis of p16. (A) and (C): Representative images from three experiments. (B) and (D): Means ± SD of three triplicate experiments. Values not sharing the same letter differ ($p < 0.05$, ANOVA, Bonferroni's post hoc test). Cells treated with 5-aza (10 μM) were positive controls.

analysis showed that treatment with 10 μM Ind resulted in a slight but significant p16^{INK4a} mRNA increase (Fig. 4b), indicating gene reactivation even under this condition. Therefore, Ind caused a specific reversal of the hypermethylation of the onco-suppressor gene *p16*^{INK4a}, accompanied by re-expression of mRNA and accumulation of 16 kD p16^{INK4a} protein.

3.3. Ind influences cell cycle progression

Working in concert with other factors, p16^{INK4a} controls cell cycle progression [38]. INK4 proteins destabilize the association of the D-type cyclin with kinase K4 or K6 (CDK4/6) thus preventing phosphorylation and inactivation of the tumor suppressor retinoblastoma protein (RB), a key regulatory step in the pathway controlling proliferation of cancer cells [39,40]. Western blot analysis showed that treatment of Caco-2 cells with 50 μM Ind for 48 h, resulted in a remarkable decrease of the hyper-phosphorylated RB, with a parallel increase of the hypo-phosphorylated form (Fig. 4a). Taken together, our data showed a significant reduction of phosphorylated RB in Ind-treated cells, consistent with a re-expressed and functional p16^{INK4a} protein. The expression of CDK4 in Ind-treated cells was comparable with that in the-untreated ones (Fig. 4a), substantiating that re-activation of RB was associated with the p16^{INK4a}-dependent CDK4 inhibition.

Inhibition of RB phosphorylation is the main mechanism to control cell cycle progression. The distribution of Caco-2 cells in different phases of the cell cycle was analyzed by flow cytometry after a 48 h treatment with 50 μM Ind, in comparison with untreated cells (Fig 4b). DNA content with PI staining showed that Ind induced a decrease of cells in G0/G1 phase, with a concurrent increase of cells in S and G2/M phases. Active hypo-phosphorylated RB has been associated with cells arrested at the G0/G1 phase [41]. Compensatory mechanisms involving other cell cycle regulators bringing about alteration of the cell cycle dynamics [42], and/or eventual activity of Ind on other check points of cell cycle, cannot be ruled out. On the other hand, it has recently been reported that p16^{INK4a} over-expression can alter cell cycle distribution of malignant cells, with a S-phase lengthening, even in the presence of a hypo-phosphorylated RB [43].

Apoptotic molecular pathways through which Ind may have affected Caco-2 cell growth were not investigated, and require other studies, however the expression of p16^{INK4a} and activation of RB may be involved in the effects observed. Besides being major controllers of the cell cycle, p16^{INK4a} and RB possess multiple anti-tumor functions [44] and their activity in regulating cell death has been shown. Over-expression of p16^{INK4a} has been associated with apoptosis of various transformed cells with down-regulation of the anti-apoptotic bcl-2 protein [45–47], and involvement of activated RB in apoptosis has been reported [48,49].

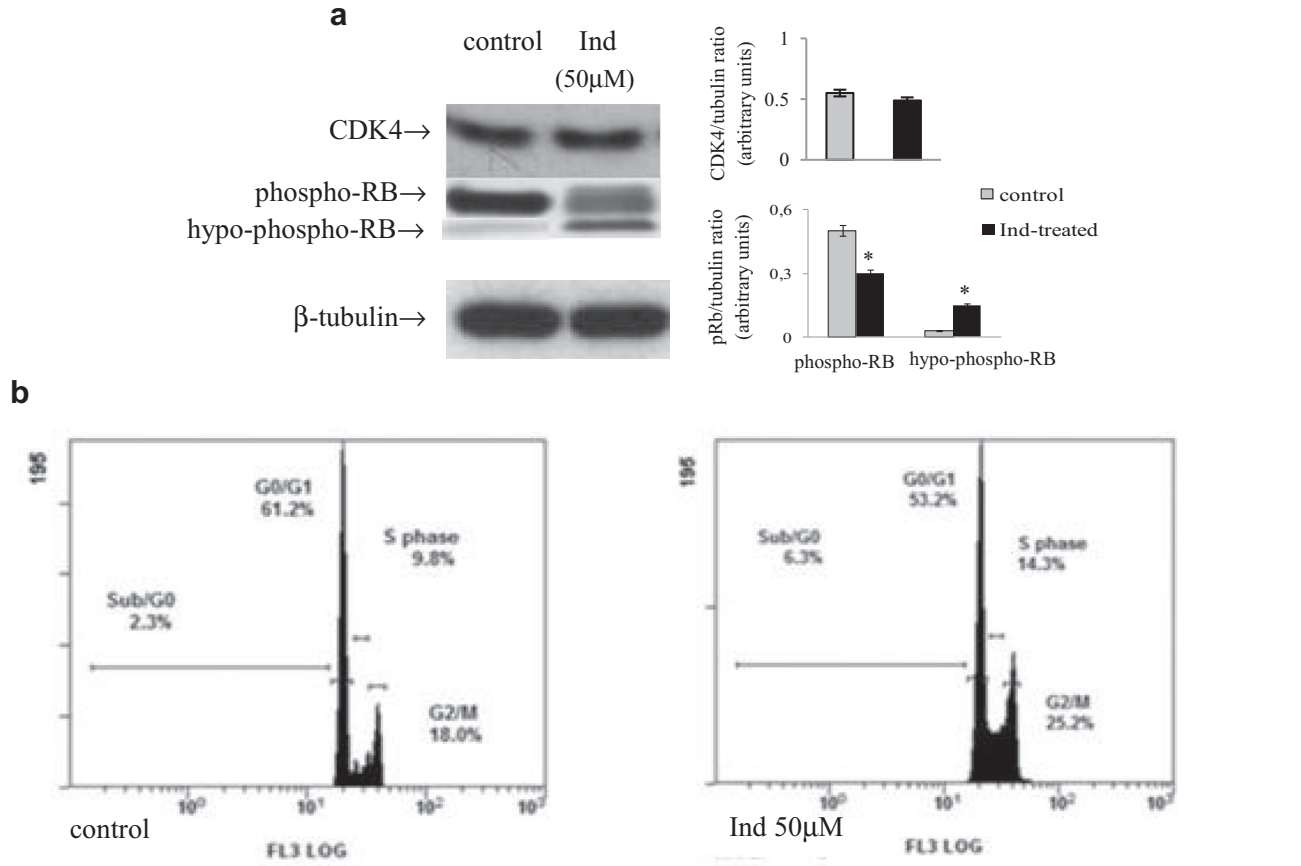

Fig. 4. Effect of Ind on (A) CDK4 and RB levels and (B) cell cycle distribution. Caco-2 cells were incubated 48 h, either in the absence (control) or in the presence of 50 μM Ind. (A) Immunoblotting and densitometric analysis of CDK4 and RB. Densitometry data are means ± SD. *significantly different from control ($p < 0.001$, Student's *t*-test). (B) Flow cytometry analysis of PI-stained cells after Ind treatment. (A) and (B) Images representative of three triplicate experiments.

Early epigenetic changes during carcinogenesis can be induced, but also reverted, by external effectors, including food components. This study, reporting for the first time that an extract from *O. ficus-indica* fruit and its pigment Ind have anti-proliferative activity in human colon cancer cells, and that Ind modulates cancer-induced epigenetic variations, stimulates research on the chemo-preventive potential of this fruit and pigment, in the same and other transformed cells. Importantly for a dietary phytochemical, Ind is scarcely degraded in the gastro-intestinal tract [19], is taken up by intestinal cells in a process not requiring metabolic transformation and exhibits high bioavailability in humans [19–21]. In this context, the amounts used are consistent with the level of Ind after dietary ingestion; when considering an intestinal volume of 600 mL [50], the solution in the digesta from a single fruit serving (149 g fruit pulp, yellow cultivar, 14.7 mg Ind) [51] may be by far higher than the amounts observed to be active.

Acknowledgments

Grant from Italy's Ministry of University and Research, MIUR (University of Palermo). MIUR had no role in the design, analysis or writing of this article.

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