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Indicaxanthin from *Opuntia Ficus Indica* (L. Mill) impairs melanoma cell proliferation, invasiveness, and tumor progression

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List of Abbrevations: AxV-FITC, annexin V-fluorescein isothiocyanate
Bcl-2, B cell lymphoma gene-2 (Bcl-2)
c-FLIP, FLICE-inhibitory protein
CXCL1, chemokine (C-X-C motif) ligand 1
MTT, 3-[4,5-*dimethyltiazol*-2-*yl*]-2,5-diphenyl tetrazolium bromide
NHEM, normal human epidermal melanocytes
NF-κB, nuclear factor kappa B
PhC, phytochemicals
PI, propidium iodide PI

ABSTRACT

Background: A strong, reciprocal crosstalk between inflammation and melanoma has rigorously been demonstrated in recent years, showing how crucial is a pro-inflammatory microenvironment to drive therapy resistance and metastasis. *Purpose:* We investigated on the effects of Indicaxanthin, a novel, anti-inflammatory and bioavailable phytochemical from *Opuntia Ficus Indica* fruits, against human melanoma both *in vitro* and *in vivo*. *Study Design and Methods:* The effects of indicaxanthin were evaluated against the proliferation of A375 human melanoma cell line and in a mice model of cutaneous melanoma. Cell proliferation was assessed by MTT assay, apoptosis by Annexin V-Fluorescein Isothiocyanate/Propidium Iodide staining, protein expression by western blotting, melanoma lesions were subcutaneously injected in mice with B16/F10 cells, chemokine release was quantified by ELISA. *Results:* Data herein presented demonstrate that indicaxanthin effectively inhibits the proliferation of the highly metastatic and invasive A375 cells as shown by growth inhibition, apoptosis induction and cell invasiveness reduction. More interestingly, *in vitro* data were paralleled by those *in vivo* showing that indicaxanthin significantly reduced tumor development when orally administered to mice. The results of our study also clarify the molecular mechanisms underlying the antiproliferative effect of indicaxanthin, individuating the inhibition of NF-κB pathway as predominant. *Conclusion:* In conclusion, we demonstrated that indicaxanthin represents a novel phytochemical able to significantly inhibit human melanoma cell proliferation *in vitro* and to impair tumor progression *in vivo*. When considering the resistance of melanoma to the current therapeutical approach and the very limited number of phytochemicals able to partially counteract it, our findings may be of interest to explore indicaxanthin potential in further and more complex melanoma studies in combo therapy, i.e. where different check points of melanoma development are targeted.

Introduction

Cancer is a growing health problem around the world and according to estimates from the International Agency for Research on Cancer (IARC), 14.1 million new cancer cases and 8.2 million cancer deaths worldwide have been reported in 2012 (Ferlay et al., 2015). By 2030, the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths simply due to the growth and aging of the population.

It has been estimated that more than two-thirds of human cancers could be prevented through modification of the lifestyle, with special attention to diet. The link between cancer risk and nutritional factors has clearly emerged in the past few years (Biesbroek et al., 2017; Bradbury et al., 2014). Specifically, both epidemiologic and basic science studies showed promising results on the effects of phytochemicals (PhC) in the chemoprevention of melanoma (Caini et al., 2017; Strickland et al., 2015; Tong and Young, 2014). Along these lines, the investigation of new molecules able to counteract the onset and/or the development of this very aggressive cancer is rapidly growing (Strickland et al., 2015).

Indicaxanthin ((2S)-2,3-dihydro-4-[2-[(2*S*)-2*a*-*carboxypyrrolidin*-1-*yl*]ethenyl]pyridine-2a,6-dicarboxylic acid), a betalain pigment from cactus pear fruit, has been the object of sound experimental work over the latest years. As many phytochemicals, indicaxanthin is a redox-active compound and has been shown to act as antioxidant in a number of *in vitro* studies (Allegra et al., 2005; Turco Liveri et al., 2009). Interestingly, thanks to its charged portions, ionizable groups and lipophilic moieties, it is amphiphilic at physiological pH (Turco Liveri et al.,

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2009) and has been demonstrated to interact with cell membranes (Tesoriere et al., 2006,2013b; Turco Liveri et al., 2009). This feature is critical to allow bioactive compounds to interact with cells and to initiate signalling events. In this regard, indicaxanthin has been showed to modulate specific redox-dependent signalling pathways involved in macrophage activation and apoptosis, epithelial and endothelial dysfunction *in vitro* (Allegra et al., 2014a; Tesoriere et al., 2013a, 2014,2015).

Remarkably, and in contrast with the majority of dietary phytochemicals, indicaxanthin is highly bioavailable (Tesoriere et al., 2004). The molecule has been shown to cross unaltered intestinal epithelial cell *in vitro* being absorbed through paracellular junctions (Tesoriere et al., 2013b). In line with that, indicaxanthin has been found in human plasma at a 7 μm peak concentration 3 h after the ingestion of four cactus pear fruits containing 28 mg of the pigment (Tesoriere et al., 2004). Moreover, its amphiphilicity allows it to cross the blood-brain-barrier and localize within the CNS (Allegra et al., 2015). Finally, thanks to its bioavailability and redox-modulating properties, indicaxanthin exerts significant pharmacological effects *in vivo*. Indeed, oral administration of the PhC at nutritionally-relevant doses (2 μmol/kg) generates, in rats, a plasma peak concentration of 0.2 μm able to exert strong anti-inflammatory effects in an *in vivo* model of acute inflammation (Allegra et al., 2014b).

The causative link between inflammation and melanoma has accurately been explored in the recent years (Bald et al., 2014; Meyer et al., 2011; Reinhardt et al., 2017; Soudja et al., 2010). Experiments in mice revealed that UV-induced skin inflammatory responses can cause the reactive proliferation and migration of melanocytes (Zaidi et al., 2011). More recently, it has been shown that reciprocal interactions between melanoma and immune cells in a pro-inflammatory microenvironment provide a source of phenotypic heterogeneity that drives therapy resistance and metastasis (Bald et al., 2014; Landsberg et al., 2012). In keeping this perspective, we decided to investigate the effects of indicaxanthin against human melanoma cell proliferation and in a model of cutaneous melanoma. We here demonstrate that indicaxanthin induces apoptosis of human melanoma cells through the inhibition of the NF-κB pathway and the downstream anti-apoptotic signalling events *in vitro* and these effects were paralleled *in vivo* in a murine model of melanoma.

Material and methods

Extraction and purification of indicaxanthin from cactus pear fruits

Indicaxanthin was isolated from cactus pear (*Opuntia Ficus-Indica*) fruits (yellow cultivar) as previously described (Allegra et al., 2014b). Briefly, the phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25. Fractions containing the pigment were submitted to cryodesiccation and purity of indicaxanthin assessed by HPLC on a Varian Microsorb C-18 column (4.6 Å~ 250 mm; Varian, Palo Alto, CA) eluted with a 20 min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a flow rate of 1.5 ml/min. Spectrophotometric revelation was at 482 nm. Under these conditions, indicaxanthin eluted after 8.15 min and was quantified by reference to standard curves constructed with 5 – 100 ng of purified compounds and by relating its amount to the peak area.

Reagents and cell culture

NHEM (Normal Human Epidermal Melanocytes) were purchased from Lonza (Walkersville, MD, USA) and grown in melanocyte growth medium 2 (Lonza). The melanoma cell lines B16/F10 and Sk-Mel-28 were purchased from IRCCS AOU San Martino – IST (Genua, Italy), A375 from Sigma–Aldrich (Milan, Italy), MALME from American Type Culture Collection (ATCC). B16/F10, A375 and Sk-Mel-28 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 μlmol/l non-essential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1 mmol/l sodium pyruvate (all from Sigma–Aldrich). MALME was cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 20% fetal bovine serum, 2 mmol/l L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1 mmol/l sodium pyruvate. Cells were grown at 37 °C in a humidified incubator under 5% CO_2. All cell lines used in this study were characterized by the cell bank of the supplier.

Proliferation assay

Cell proliferation was measured by the 3-[4,5-*dimethyltiazol*-2-*yl*]-2,5-diphenyl tetrazolium bromide (MTT). Briefly, the human melanoma cells and the NHEM cells were seeded on 96-well plates (2×10^3 cells/well) and treated with indicaxanthin (50-100-200 μm) for 72 h before adding 200 μl of MTT (Sigma, Milan, Italy) (0,25 mg/ml in medium). Cells were thus incubated for an additional 3 h at 37 °C. After this time interval, cells were lysed, and dark blue crystals were solubilized with 100 μl of dimethyl sulfoxide (DMSO). The optical density of each well was measured with a microplate spectrophotometer (TitertekMultiskan MCC/340), equipped with a 490 nm filter.

Apoptosis assay

The externalization of phosphatidylserine to the cell surface was detected by flow cytometry using a double-staining with Annexin V-Fluorescein Isothiocyanate (AxV-FITC) and Propidium Iodide (PI) as previously reported (Tesoriere et al., 2013a). Briefly, cells were seeded in triplicate in 24-well plates at a density of 2.0×10^5 cells/cm^2. After an overnight incubation, they were washed with fresh medium and incubated with indicaxanthin at 100 μm. After 48 h, cells were harvested by trypsinization and adjusted at 2.0×10^5 cells/ml with combining buffer. One hundred μl of cell suspension was then incubated with 5 μl of a 5 μm AxV-FITC solution and 10 μl of a 20 μg/ml PI solution, at room temperature in the dark for 15 min. Samples of at least 1.0×10^4 cells were then analysed by Epics XL flow cytometer using Expo32 software (Beckman Coulter, Fullerton, CA) and an appropriate bi-dimensional gating method.

Western blot analysis

A375 cells were treated with indicaxanthin 100 μm for 15-30-60 min or 6-24-48 h. Whole-cell or nuclear extracts were prepared as previously described (Panza et al., 2015). The protein concentration was measured by the Bradford method (Bio-Rad, Milan, Italy). Equal amounts of protein (40 μg/sample) from whole or nuclear cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Trans-Blot Turbo Transfer Starter System, Biorad). The membranes were blocked for 2 h in 5% low-fat milk in PBS with 0.1% Tween 20 (PBST) at room temperature. Then the filters were incubated with the following primary antibodies: Caspase-3 and NF-κB p65 (Cell Signaling, USA; diluted 1:1000), IκBα (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:200); LC3B (Novusbio, USA; diluted 1:1000); Bcl-2 (Cell Signaling, USA; diluted 1:1000), c-FLIP (Millipore; diluted 1 μg/ml), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:1000), GAPDH (Cell Signaling, USA; diluted 1:1000), α-Tubulin (Cell Signaling, USA; diluted 1:1000) overnight at 4 °C. The membranes were washed 3 times with PBST and then incubated with horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:2000) for 2 h at room temperature. The immune complexes were visualized by the ECL chemiluminescence method and acquired by the Image Quant 400 system (GE Healthcare).

Invasion assay

The assay was performed using chambers with polycarbonate filters with 8 μm nominal pore size (Millipore, USA) coated on the upper side with Matrigel (Becton Dickinson Labware, USA) as previously described (Panza et al., 2016). Briefly, the chambers were placed into a 24-well plate and melanoma cells (2.5×10^5/ml) were plated in the upper chamber, with or without indicaxanthin 100 μm, in serum-free DMEM. After the incubation period (16 h), the filter was removed, and non-migrant cells on the upper side of the filter were detached with the use of a cotton swab. Filters were fixed with 4% formaldehyde for 15 min, and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and then washed with PBS. The filters were examined microscopically and cellular invasion was determined by counting the number of stained cells on each filter in at least 4–5 randomly selected fields. Data are presented as a mean of invaded cells ± SD/microscopic field of three independent experiments.

Animals

Animal care was in accordance with Italian and European regulations on the protection of animals used for experimental and other scientific purposes. Mice were observed daily and humanely euthanized by CO_2 inhalation if a solitary subcutaneous tumor exceeded 1.5 cm in diameter or mice showed signs referable to metastatic cancer. All efforts were made to minimize suffering. Male C57BL/6 mice (age 6–7 weeks, 18–20 g) were from Charles River Laboratories, Inc. Mice were housed at the Animal Research Facility of the Department of Pharmacy of the University of Naples Federico II.

Induction of subcutaneous B16 lesions

Mice were subcutaneously (s.c.) injected in the right flank with B16/F10 cells (1×10^5/0.1 ml) and divided in two groups. The treated group received indicaxanthin (3.2 mg/kg) orally three time a day for 14 days. Control mice received only vehicle. Tumor size was measured using a digital caliper, and tumor volume was calculated using the following equation: tumor volume = π/6(D1xD2xD3) where D1 = length; D2 = width; D3 = height and expressed as cm^3.

ELISA

CXCL1/KC plasma concentrations were evaluated using ELISA kits according to the manufacturer's instruction (DuoSet ELISA, R&D systems, Minneapolis, MN, USA).

Statistical analysis

Data from all experiments are reported as mean ± SEM unless otherwise noted. Data were analysed and presented using GRAPHPAD PRISM software (GraphPad). Significance was determined using Student's 2-tailed *t* test. Results were considered significant at *P* values less than 0.05 and are labelled with a single asterisk. In addition, *P* values less than 0.01 and 0.001 are designated with double and triple asterisks, respectively.

Results and discussion

Indicaxanthin suppresses human melanoma cell proliferation

Indicaxanthin was purified as above reported and its HPLC-based chemoprofile along with the chemical structure are shown in Fig. 1.

The antiproliferative effect of indicaxanthin on human (A375, Sk-Mel-28, MALME) or murine (B16F10) melanoma cells was evaluated by MTT assay.

Incubation of cells with indicaxanthin (50-100-200 μm) for 72 h, caused a concentration-dependent inhibition of all cell lines used (Table 1). The most sensitive cell lines to the inhibitory effect of indicaxanthin were A375 and the B16F10, whose proliferation was inhibited by 21%, 36%, 56% and 34%, 46%, 69%, respectively (Table 1). On the other hand, NHEM growth was not affected by any of the concentration of indicaxanthin tested at the time point considered (data not shown), thus indicating a selective cytotoxic activity toward cancer cell lines. Curcumin was used as positive control (Table 1).

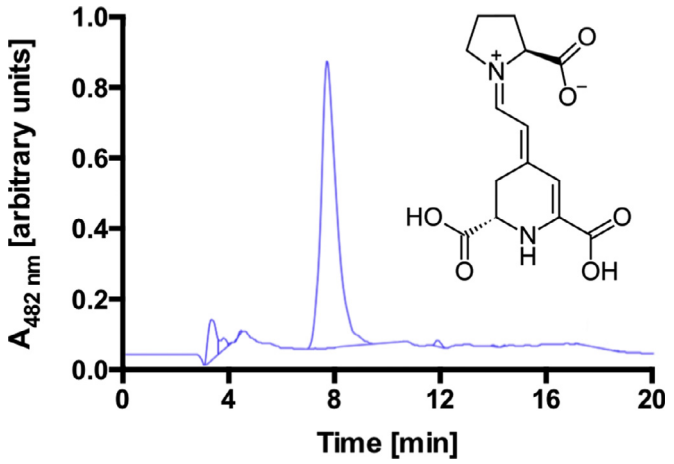

Fig. 1. Representative chromatographic profile of indicaxanthin used in the present study; in the inset the chemical structure of the phytochemical.

Table 1
Effect of indicaxanthin (IND) on A375, Sk-Mel-28, MALME and B16/F10 melanoma cells proliferation. Growth inhibition was measured by MTT assay and is expressed as percent of inhibition at 72 h. Curcumin (CUR) was used as positive control. Experiments were run in triplicate, each performed in quadruplicate (***$P < 0.001$ vs. untreated cells).

	IND 50 μm	IND 100 μm	IND 200 μm	CUR 100 μm
A375	20.7***	35.7***	56***	73.3***
Sk-Mel-28	4.2	4.7	24.4 ***	65.2***
MALME	4.9	12.9	30.8 ***	69.4***
B16/F10	33.9***	46.4***	69.2***	70.2***

Indicaxanthin induces apoptosis of human melanoma A375 cell. Cell death can be mediated by several intracellular programs

To gain insights into the mechanisms of indicaxanthin-induced cell growth inhibition (necrosis or apoptosis), externalization of plasma membrane phosphatidylserine, a reliable marker of cell apoptosis, was evaluated for A375 cell line at 48 h. The choice of this cell line was prompted by its high sensitivity to indicaxanthin effect (see Table 1). The concentration of the phytochemical chosen for this study (100 μm), was selected taking into account the value of GI_{50} measured for this cell line.

As shown in Fig. 2A and B, treatment of A375 cells with indicaxanthin induced a significant increase of the percentage of apoptotic/necroptotic cells (AxV-$FITC^+$/PI^+, [31.5 ± 0.2] % ($P < 0.01$) as compared to untreated cells. On the other hand, indicaxanthin treatment determined only neglectable necrotic events as evaluated by the percentage of AxV-$FITC^-$/PI^+ cells ([1.3 ± 0.01] %, Fig. 2A). As caspase-3 is the final stage of apoptosis initiation, we also examined its activity. Results obtained showed that treatment with indicaxanthin markedly promoted cleavage and subsequent activation of caspase-3 (Fig. 2C). However, melanoma is one type of cancer that constantly develops drug resistance due to dysregulation of apoptosis (Grossman and Altieri, 2001). Therefore, the induction of other forms of cell death, especially autophagy, is necessary and fundamental to

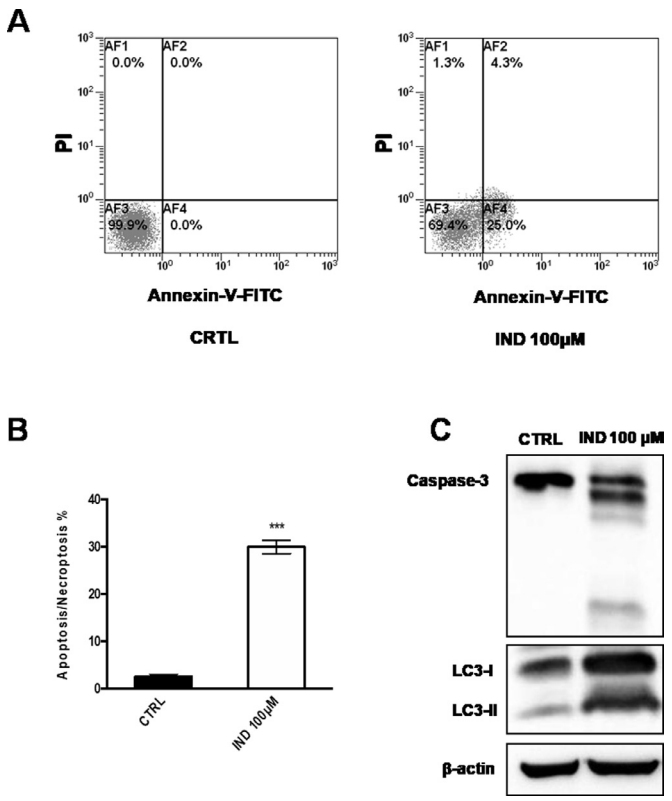

Fig. 2. Effect of indicaxanthin on A375 melanoma cells apoptosis. (A) Cells were incubated for 48 h in the absence (CTRL) or presence of indicaxanthin (IND) 100 μm and apoptosis was determined by Annexin V-FITC/Propidium Iodide (PI) staining. AF3, viable cells (Annexin V-FITC$^-$/PI$^-$); AF4, early apoptotic cells (Annexin V-FITC$^+$/PI$^-$); AF2, tardive apoptotic/necroptotic cells (Annexin V-FITC$^+$/PI$^+$); AF1, necrotic cells (Annexin V-FITC$^-$/PI$^+$). Images are representative of three experiments with comparable results. (B) Quantitative analysis of indicaxanthin-induced A375 cell apoptosis at 48 h. Experiments were run in triplicate, each performed in quadruplicate (**$P < 0.01$; ***$P < 0.001$ vs. CTRL). (C) Analysis of caspase 3 activation and LC3II/I conversion in A375 cells treated with IND 100 μm by immunoblotting. β-actin was used as a loading control.

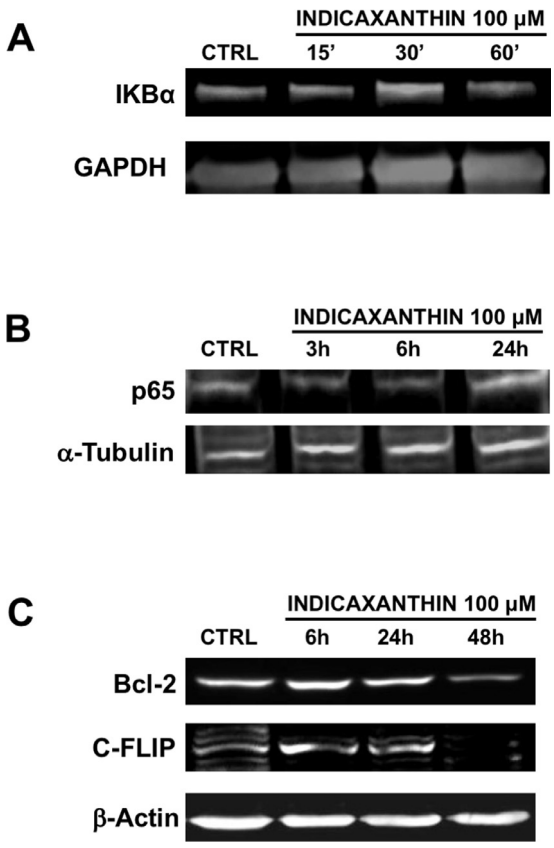

Fig. 3. Effect of indicaxanthin on NF-κB activation and NF-κB-dependent anti-apoptotic proteins in A375 human melanoma cells. (A) Western blot analysis of IκBα carried out on the cytosolic extracts obtained from A375 cells incubated in the absence or in the presence of indicaxanthin 100 μm for 15, 30, or 60 min; (B) western blot analysis of p65 carried out on nuclear extracts obtained from A375 cells incubated in the absence or in the presence of indicaxanthin 100 μm for 6–24 h; (C) western blot analysis of Bcl-2 and C-FLIP carried out the cytosolic extracts obtained from A375 cells incubated in the absence or in the presence of indicaxanthin 100 μm for 6–48 h. GADPH, α-tubulin and β-actin were detected as a loading control. Images are representative of three experiments with comparable results.

conquer this resistance. To gain further insights into the mechanisms of indicaxanthin-induced cell growth inhibition A375 cells were treated with indicaxanthin (100 μm) and autophagy was evaluated by measuring the conversion of LC3-I to LC3-II, a sign of autophagic activity (Mizushima et al., 2010). As shown in Fig. 2C, 100 μm indicaxanthin treatment caused a marked increase of LC3-II level in A375 cells as compared to control. These results revealed that indicaxanthin could potently induce both apoptosis and autophagy in A375 cells.

Caspase-8 and autophagy are apparently gate keepers preventing necroptosis (Kaczmarek et al., 2013). Since caspase-3 is activated downstream of caspase-8 in the death-receptor pathway we can exclude an involvement of necroptosis in indicaxanthin-induced cell death.

Indicaxanthin inhibits NF-κB activation and regulates the expression of NF-κB-dependent anti-apoptotic proteins in A375 human melanoma cells

Nuclear factor kappa B (NF-κB) signalling pathway is a complex network linking extracellular stimuli to cell survival and proliferation. NF-κB is, indeed, one of the major transcription factors associated with the development and progression of both hematologic and solid tumours, including melanoma, breast, prostate, ovarian, pancreatic, colon, lung, and thyroid cancers. Aberrantly activated NF-κB plays a fundamental role in cell survival, resistance to apoptosis (Madonna et al., 2012), angiogenesis, tumor cell invasion, metastasis (Basseres and Baldwin, 2006) and chemoresistance (Fujioka et al., 2012). Along these lines, NF-κB is currently considered an ideal target for cancer therapy. NF-κB is normally retained in an inactive form in the cytoplasm tightly associated to the inhibitory protein IκB-α.

In order to investigate indicaxanthin effects on NF-κB activity, A375 cells were treated with indicaxanthin (100 μm) and western blot analysis was carried out on cellular extracts obtained at different time points (15-30-60 min and 3-6-24-48 h). As shown in Fig. 3A indicaxanthin inhibited IκBα degradation in A375 cells as demonstrated by an increase in band intensity at 30 min. This effect was paralleled by a reduction in the nuclear levels of the NF-κB active subunit p65, at 3 and 6 h after incubation with indicaxanthin (Fig. 3B).

To further confirm that indicaxanthin-induced apoptosis of melanoma cells was strictly related to NF-κB-inhibition western blot experiments were carried out to evaluate the effect on the expressions of two anti-apoptotic proteins, B cell lymphoma gene-2 (Bcl-2) and FLICE-inhibitory protein (c-FLIP) whose transcription is modulated by NF-κB. As shown in Fig. 3C, A375 cells incubated with indicaxanthin exhibited a marked reduction in both proteins band intensity.

Our data showing the ability of indicaxanthin to inhibit NF-κB activation provide a molecular insight of the mechanism through which the PhC could impair cell proliferation and stimulate apoptosis. Moreover, it gains a remarkable interest in the light of the current great interest of researchers and pharmaceutical companies in the

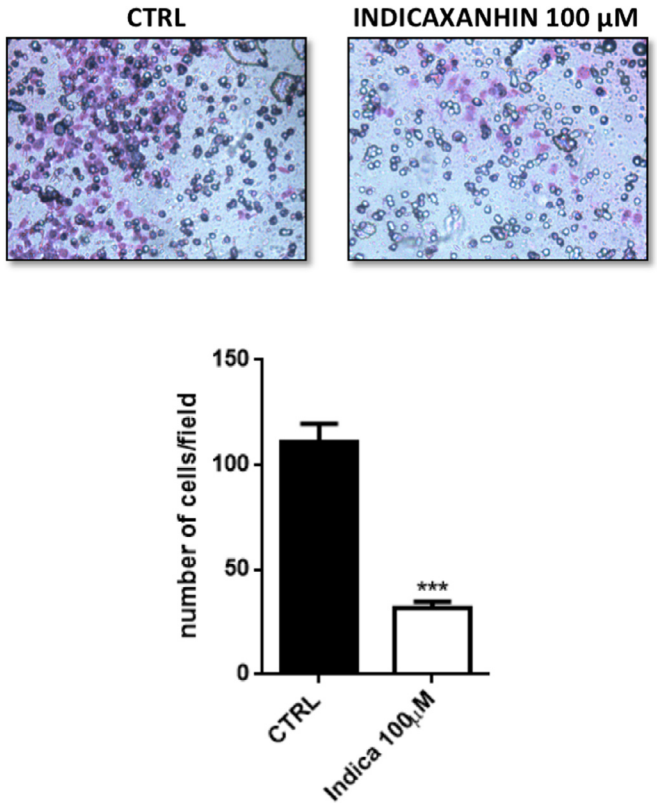

Fig. 4. Effect of indicaxanthin on the invasiveness of A375 cells. Indicaxanthin (100 µm) suppresses A375 cell invasion as measured by a transwell cell invasion assay. Representative field of invasive cells A375 on the membrane and relative average number of invasive cells from triplicate measurements. (***$P < 0.001$ vs. CTRL).

identification of new molecules able to modulate NF-κB pathway. Over 750 inhibitors of the NF-κB pathway have been identified and a lot of them are natural compounds (Gilmore and Herscovitch, 2006). Therefore, novel dietary supplements to inhibit NF-κB activation may offer a very promising option in melanoma treatment.

Effect of indicaxanthin on cells invasion

To determine whether indicaxanthin affected the invasive ability of the metastatic melanoma cells A375, we performed a cell invasion assay using a transwell system. As shown in Fig. 4, indicaxanthin 100 µm (a concentration not affecting cell proliferation at 16 h) significantly inhibited the invasiveness of A375 cell line by 71% ($P < 0.001$ *vs* control; $n = 3$).

Indicaxanthin inhibits growth of melanoma tumors in vivo by reducing plasma levels of melanoma-associated chemokines

Finally, to confirm the antitumor capacity of indicaxanthin we examined its effects on tumor growth *in vivo*. To this end, we used a murine model of cutaneous melanoma obtained by the subcutaneous injection of B16F10 murine melanoma cells in C57BL/6 mice.

Tumor-bearing mice were treated with indicaxanthin (3.2 mg/kg) or vehicle (saline) by oral gavage twice daily. This dose was 5-times higher than that employed in previous *in vivo* experiments investigating the anti-inflammatory effects of the PhC (0.65 mg/kg) (Allegra et al., 2014b). At day 14 after tumor implantation, a reduction in tumor volume (86%) and weight (83%) was observed in indicaxanthin-treated mice (0.035 ± 0.01 cm^3 mean tumor volume vs. control mice 0.260 ± 0.04 cm^3 mean tumor volume, $P < 0.001$) (Fig. 5A and B). Moreover, in the plasma of indicaxanthin-treated mice we found a significant reduction of CXCL1 levels by 42% (140 ± 17 pg/ml; $P < 0.05$) as compared to control mice (240 ± 28) (Fig. 5C). CXCL1 is a chemokine belonging to the CXC chemokine subfamily and it has been associated with metastatic melanoma since it facilitate the recruitment of tumor promoting myeloid cells into the tumor and enhances angiogenesis (Richmond, 2010).

As above stated, indicaxanthin is a bioavailable PhC in rats where it reaches a therapeutical plasma concentration of 0.22 µm after an oral administration of 0.64 mg/kg (Allegra et al., 2014b). Taking into account that we here administered a 5-time higher amount of the PhC in mice, we estimate that the plasma concentration of indicaxanthin in this study may well be close to this value.

Indicaxanthin is, however, a thermo-unstable compound: 50% of the molecule is lost after a 24 incubation at 37 °C (not shown). Taking all this into account, we then believe that the antiproliferative effects observed in the present study should be induced by a concentration of

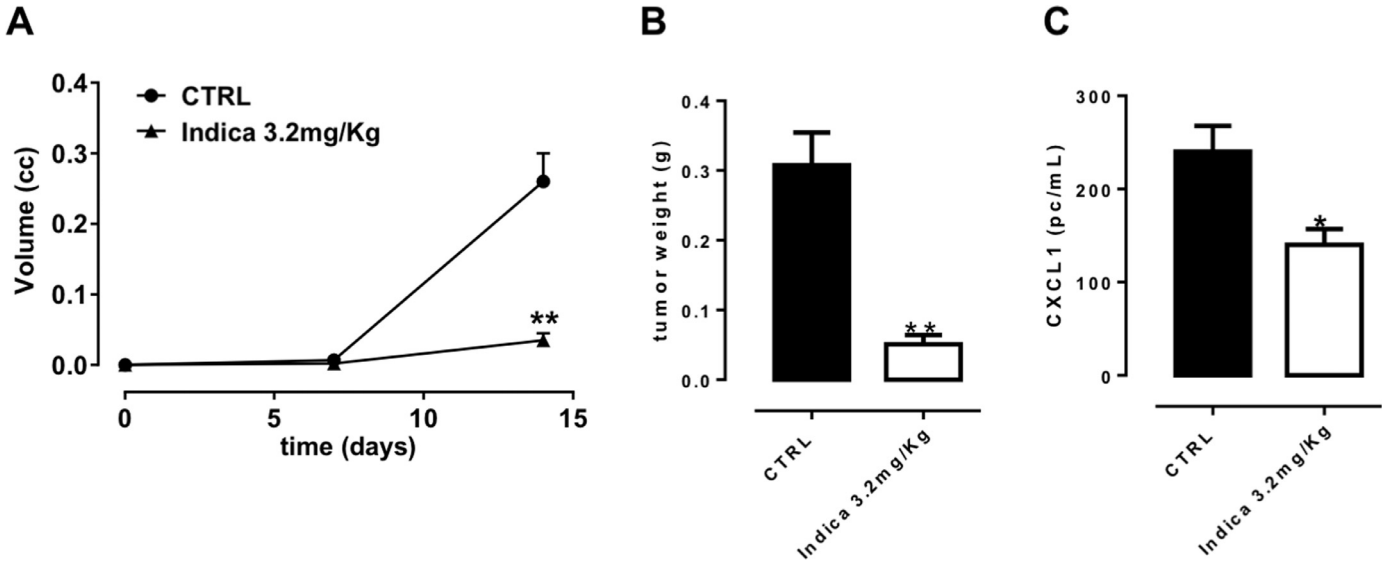

Fig. 5. Effect of indicaxanthin on tumor growth *in vivo* and plasma levels of the chemokine CXCL1. Indicaxanthin (3.2 mg/kg) was given orally to mice, while control mice received vehicle only. (A) the average tumor volume with standard error is plotted against the days after tumor implant; (B) tumor weight and (C) CXCL1 levels in control and indicaxanthin-treated mice. (*$P < 0.05$ vs. CTRL; **$P < 0.01$ vs. CTRL).

the molecule in a range much lower than above mentioned GI_{50} (100 μm) and more likely between 50–12.5 μm.

Conclusions

In conclusion, we demonstrated that indicaxanthin represents a novel phytochemical able to significantly inhibit human melanoma cell proliferation *in vitro* and markedly impair tumour progression *in vivo*.

The retention of the antitumoral effects of indicaxanthin *in vivo* and its ability to interact with a key molecular target in the etiopathogenesis of melanoma, i.e. NF-κB, appear of interest taking into account the limited and inadequate number of pharmacological agents for melanoma treatment. In this regard it is important to underlie how the complexity and aggressiveness of melanoma made it hardly to be controlled with just one pharmacological/phytotherapeutical agent. Along these lines we propose indicaxanthin as a novel therapeutical agent to be further explored in more complex studies in combo therapy, i.e. with other therapeutical agents targeting different check points of melanoma development.

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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