



Original Article

Zeaxanthin from *Porphyridium purpureum* induces apoptosis in human melanoma cells expressing the oncogenic BRAF V600E mutation and sensitizes them to the BRAF inhibitor vemurafenib



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ABSTRACT

Zeaxanthin, an abundant carotenoid present in fruits, vegetables and algae was reported to exert antiproliferative activity and induce apoptosis in human uveal melanoma cells. It also inhibited uveal melanoma tumor growth and cell migration in nude mice xenograft models. Here we report that zeaxanthin purified from the rhodophyte *Porphyridium purpureum* (Bory) K.M.Drew & R.Ross, Porphyridiaceae, promotes apoptosis in the A2058 human melanoma cell line expressing the oncogenic BRAF V600E mutation. Zeaxanthin 40 μM (IC_{50}) induced chromatin condensation, nuclear blebbing, hypodiploidy, accumulation of cells in sub-G1 phase, DNA internucleosomal fragmentation and activation of caspase-3. Western blot analysis revealed that zeaxanthin induced up-regulation of the pro-apoptotic factors Bim and Bid and inhibition of NF- κB transactivation. Additionally, zeaxanthin sensitized A2058 melanoma cells *in vitro* to the cytotoxic activity of vemurafenib, a BRAF inhibitor widely used for the clinical management of melanoma, suggesting its potential interest as dietary adjuvant increasing melanoma cells sensitivity to chemotherapy.

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Introduction

Melanomas account for less than 2% of skin cancers but are responsible for 80% of their mortality (Armstrong and Krickler, 2001; Grange, 2005; Leiter and Garbe, 2008; MacKie et al., 2009). Melanoma cells are characterized by mutations that confer them a strong resistance to anticancer drugs-induced apoptosis and selective advantages for cell survival, proliferation and metastasis (Locatelli et al., 2013). Particularly, mutations in the BRAF oncogene are found in 70% of malignant melanoma (Davies et al., 2002; Haluska et al., 2006; Dankort et al., 2009; Dutton-Regester et al., 2012; Jang and Atkins, 2014) and lead to over-activation of the MAP kinase pathway that stimulates cell proliferation. Most anticancer drugs only delay the early growth of melanoma tumors but fail to provide a long-term cure because of the rapid acquisition of drug resistance (Locatelli et al., 2013; Spagnolo et al., 2014).

Additionally, melanoma cells display pronounced neoangiogenesis and a high ability to escape immune cell that explain why the 5-year survival rate for metastatic melanoma ranges from 5 to 10%, with a median survival of less than 8 months (Marneros, 2009; Mathieu et al., 2012). Brain metastasis are present in 75% of advanced melanoma patients and constitute a major cause of mortality because of the low permeability of the blood-brain barrier to chemotherapeutic drugs (Hall et al., 2000). The search for cytostatic, antimetastatic and antiangiogenic molecules in plants and algae has established that carotenoids have a great potential as natural antimelanoma compounds (Hashimoto et al., 2011; Tanaka et al., 2012; Firdous et al., 2010; Gagez et al., 2012; Baudelet et al., 2013; Reboul, 2013; Chung et al., 2013; Kim et al., 2013; Xu et al., 2015; Lu et al., 2015; Chen et al., 2017). These pigments have no oral toxicity, are resorbed by enterocytes, transported in blood after *per os* consumption (Burri et al., 2001; Hashimoto et al., 2011; Reboul, 2013) and can integrate cell membranes (Reboul, 2013; Oliveira-Junior et al., 2016) and reach tumor cells where they exert cytotoxic, cytostatic, antimetastatic, anti-inflammatory and antiangiogenic activities (Sugawara et al., 2006; Gagez et al., 2012).

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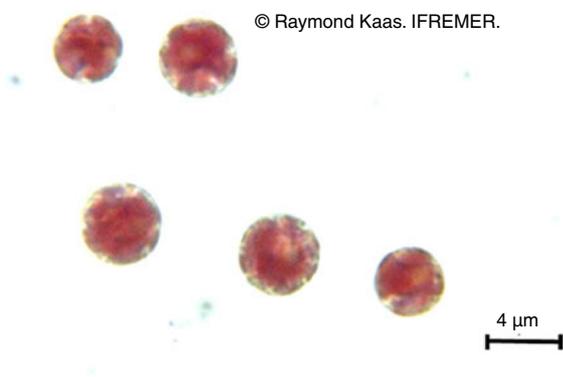


Fig. 1. *Porphyridium purpureum* strain CCAP 1380.3. © Raymond Kaas. IFREMER.

Fucoxanthin, a carotenoid present in brown microalgae and seaweeds inhibits melanoma cells and tumor growth *in vitro* and *in vivo* (Chung et al., 2013; Kim et al., 2013). It also limits melanoma metastasis in murine models (Chung et al., 2013; Kumar et al., 2013), suggesting that it also has a clinical efficacy in humans. We recently demonstrated that zeaxanthin (**1**), an abundant carotenoid found in various dietary sources (corn, spinach, saffron, seaweeds, microalgae) inhibits the *in vitro* growth of the highly invasive human melanoma cell line A2058 (Baudelet et al., 2013). Zeaxanthin also induced apoptosis in two human uveal melanoma cell lines (SP6.5 and C918) without impairing the cell viability of non cancer uveal melanocytes (Bi et al., 2013; Xu et al., 2015). Zeaxanthin-induced apoptosis was associated to a decrease in the expression of the antiapoptotic proteins Bcl-2 and Bcl-xL and an increase in the expression of the proapoptotic proteins Bak and Bax (Bi et al., 2013). Zeaxanthin also evoked the release of mitochondrial cytochrome c in the cytosol and caspase-9 and -3 activation (Bi et al., 2013). In the present report, we performed additional experiments to further elucidate the molecular mechanisms of zeaxanthin pro-apoptotic activity in melanoma cells and assessed its ability to sensitize melanoma cells to a BRAF inhibitor used to treat clinical melanoma. We selected the highly invasive A2058 human melanoma cell line as a relevant clinical model expressing the V600E BRAF oncogenic mutation (Dutton-Regester et al., 2012).

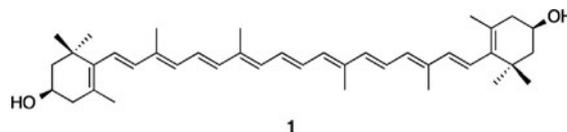
Material and methods

Microalgae culture, harvest and freeze-drying

Porphyridium purpureum (Bory) K.M.Drew & R.Ross CCAP 1380.3 (bangiophyceae, rhodophyte) (Fig. 1) was grown at $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance. Cells were grown in four units of 50-l column photobioreactors with 35‰ salinity seawater enriched by Walne medium (Walne, 1966; Juin et al., 2015). Batch cultures were maintained at 20 °C under continuous light provided by fluorescent lamps (Philips TLD 58W 865) and bubbled with 0.22 μm filtered air containing 3% CO₂ (v/v). Microalgae were harvested after 12–16 days of growth and separated from the culture medium by a two-step process. First step used a clarifier separator (Clara 20, Alfa Laval Corporate AB, Sweden) at 100 l h⁻¹, 9000 × g, at room temperature. Step two used a soft centrifugation at 4000 × g, 20 min, at 4 °C to separate the slurry. Algal paste was freeze-dried at –55 °C and P < 1 hPa, on a freeze-dryer equipped with a HetoLyopro 3000 condenser and a Heto cooling trap (Thermo, France).

Purification and characterization of *Porphyridium purpureum* zeaxanthin

Porphyridium purpureum pigments were extracted in ethanol using a mixer mill extraction process developed in our group (Serive et al., 2012). Zeaxanthin (**1**) identification was confirmed after separation by analytical RP-HPLC (Zapata et al., 2000) and cross-check analysis of its polarity, absorption spectrum, maximal absorption wavelengths, band III/II ratio and fragmentation profile in UPLC-MS^E (Roy et al., 2011; Baudelet et al., 2013; Juin et al., 2015), in comparison with standard zeaxanthin (Sigma–Aldrich, France). Pure zeaxanthin was then collected by preparative RP-HPLC (Pasquet et al., 2011) in glass vials, dried under reduced pressure in a Buchi R-210 rotatory evaporator at 40 °C (Buchi, France) and stored at –80 °C before use in cell culture experiments.



Cell culture

A2058 (ATCC[®] CRL-11147TM, LGC ATCC Standards, France) is a melanoma cell line established from metastatic cells removed from the lymph node of a 43 years old male caucasian patient. It constitutes a clinically relevant model to assess the cytotoxicity of new antimelanoma drugs as it combines high invasive, metastatic and chemoresistance potentials with a gene mutation profile often encountered in human melanomas (V600E mutation in BRAF and mutations in the PTEN and P53 genes) (Dankort et al., 2009). Cells were routinely grown as monolayers, at 37 °C in a 5% CO₂–95% air humidified atmosphere, in DMEM (Fischer scientific, France) supplemented with 10% heat-inactivated (56 °C, 30 min) FCS (Dutscher, France) to which were added penicillin 100 U ml⁻¹ and streptomycin 100 $\mu\text{g ml}^{-1}$.

Determination of zeaxanthin IC₅₀ in A2058 melanoma cells

Purified zeaxanthin (**1**) was solubilized in DMSO at 6 mM (stock solution) and diluted in cell culture medium to obtain 5–60 μM solutions. The final DMSO concentration in the cell culture medium was lower than 1%, tested as a negative control and validated as a non cytotoxic concentration. The antiproliferative activity of zeaxanthin was determined using the MTT assay (Sigma–Aldrich, France) as previously described (Putey et al., 2007; Baudelet et al., 2013; Hedidi et al., 2016). IC₅₀ was determined using the free Graph pad Prism software using the “sigmoidal dose response” (variable slope) function.

Nuclear membrane modification, chromatin condensation and DNA fragmentation

Sub-confluent A2058 melanoma cells were trypsinized and 2×10^5 cells were seeded in 6-well plates, in a final volume of 3 ml of control medium or culture medium containing zeaxanthin 40 μM (IC₅₀) or staurosporine 2 μM . Cells were grown for 72 h at 37 °C and washed in PBS 0.1 M pH 7.4, before being fixed with formaldehyde 3% for 30 min at 37 °C. Cells were then rinsed in PBS, permeabilized with Triton X-100 1% in PBS and stained with DAPI 2 $\mu\text{g ml}^{-1}$ for 1 h at 37 °C. Cells were rinsed, mounted on glass microscope slides and observed using a Leica epifluorescence microscope equipped with an epifluorescence A filter block (excitation 340–360 nm) and a numeric camera.

Annexin V-Cy3 and 6-CFDA detection assay

Apoptosis was evaluated by using double staining with Annexin V-Cy3 (red) and 6-carboxyfluorescein diacetate (6-CFDA, green) (Sigma–Aldrich®, France). Cells (5×10^3 cells/well) were incubated in conventional culture conditions for 24 h. Then, cells were treated with zeaxanthin (IC_{50} , $40 \mu\text{M}$) for 72 h and staurosporine (positive control, $1 \mu\text{M}$) for 24 h. Cells were further washed with PBS, suspended in binding buffer and stained with Annexin V and 6-CFDA solution for 10 min. DAPI solution was also added to the wells for DNA labelling. Finally, cells were observed under fluorescence microscope (ZEISS Axio Observer).

Flow cytometric detection of apoptotic cells

Melanoma cells were grown in control culture medium or treated with zeaxanthin $40 \mu\text{M}$ for 72 h before being stained for 30 min at 37°C in PBS containing propidium iodide ($PI 100 \mu\text{g ml}^{-1}$) and Rnase A ($100 \mu\text{g ml}^{-1}$) (Molecular Probes, France). Cells were washed and suspended in 1 ml PBS before being analyzed using a FACS Cantoll flux cytometer (BD Biosciences, France) equipped with an air cooled blue LASER ($\lambda = 488 \text{ nm}$, 20 mW). Light diffusion parameters (forward and lateral scatter lights) were optimized to define the size threshold excluding cellular debris and cell clusters for single-cell fluorescence analysis. PI fluorescence was measured using a FL3 filter ($\lambda \equiv 670 \text{ nm}$) and analyzed using the BD FACS Diva Software (BD Biosciences, France). Distribution of melanoma cells in the different cell cycle phases and hypodiploidy was determined according to their DNA content as measured by the fluorescence intensity of PI: Diploid cells ($2n$): G0/G1 phase; Replicative cells ($2n < \text{DNA content} < 4n$): S Phase; Tetraploid cells ($4n$): G2/M phase; hypodiploid cells (DNA content $< 2n$): apoptotic Sub-G1 Phase.

DNA internucleosomal fragmentation

After treatment with control culture medium or zeaxanthin $40 \mu\text{M}$, 10^6 melanoma cells were washed with PBS and lysed in $400 \mu\text{l}$ lysis buffer (Tris–HCl 10 mM pH 8 NaCl 150 mM, EDTA 40 mM, SDS 1%, proteinase K 0.2 mg ml^{-1}) for 3 h at 56°C . The cell lysate was centrifuged ($11,000 \times g$, 15 min, 4°C) and the DNA contained in the supernatant was extracted for 15 min using a mix of phenol/chloroform/isoamyl alcohol (PCI) (25/24/1, v/v/v) at pH 9. The mix was centrifuged ($11,000 \times g$, 15 min, 4°C), and the aqueous phase was collected to precipitate DNA using sodium acetate 3 M in 1 ml absolute ethanol (1 night, -20°C). The precipitated DNA was centrifuged ($11,000 \times g$, 30 min, 4°C), the supernatant was discarded and the pellet was dried 5 min at 60°C . The DNA pellet was suspended in $50 \mu\text{l}$ Ultrapure water containing RNase A $100 \mu\text{g ml}^{-1}$ for 30 min at 37°C . Twenty microliters of the DNA extract were loaded and separated on an agarose/tris-borate-EDTA 1% gel for 30 min at 100 V. Gels were stained with ethidium bromide, and observed using a UV transilluminator.

Caspase-3 colorimetric assay

Caspase-3 activation was quantified using a commercial assay based on the hydrolysis of Ac-DEVD-pNA (CASP3C kit, Sigma–Aldrich, France).

Western-blot

A2058 cells were incubated in control culture medium or in the presence of zeaxanthin (**1**) $40 \mu\text{M}$ for 72 h. The cells were collected and lysed in a lysis buffer (HEPES 50 mM pH 7.4 CHAPS 5 mM DTT 5 mM). Total proteins were separated by 10% SDS-PAGE

and then transferred to a nitrocellulose membrane. The membranes were blocked with 5% (w/v) nonfat dry milk in TBS for 1 h and changed to an appropriate dilution of specific primary antibodies against Bid, Bim, Bak, Bcl-xL, $I\kappa\text{B}\alpha$, NF- κB p65, p-NF- κB (Ser536), IKK α , IKK β and β -actin (Ozyme, France) in milk overnight at 4°C . The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit IgG (1:5000). Signals were detected using the ChemiDoc™ imaging system (Biorad, France).

Sensitization of A2058 melanoma cells to the BRAF inhibitor Vemurafenib

Vemurafenib was obtained from Selleckchem, France, diluted to a 10 mM stock solution in PBS 0.1 M pH 7.4, before further dilution in cell culture medium. A2058 cells were incubated for 72 h in control culture medium or in the presence of zeaxanthin $40 \mu\text{M}$, Vemurafenib (0.1, 1 and $5 \mu\text{M}$) or in a mix of zeaxanthin $40 \mu\text{M}$ and Vemurafenib (0.1, 1 and $5 \mu\text{M}$). Antiproliferative activity was calculated using the MTT assay and potentiation of the Vemurafenib antiproliferative activity by zeaxanthin was expressed as the percentage of growth inhibition increase as compared to Vemurafenib alone.

Statistics

Antiproliferative activity of zeaxanthin was expressed as percentage growth inhibition \pm SEM from three independent assays. The normal distribution of absorbance data in control and treated cells was demonstrated using the Hartley's Fmax test to confirm homogeneity of absorbances variances. The statistical significance of proliferation differences between control and treated cells was then investigated by an unpaired Student's *t* test, using a free online calculator developed by Institut Pierre Louis d'Epidémiologie et de Santé publique UMR S 1136 INSERM University Pierre et Marie Curie Paris (<http://marne.u707.jussieu.fr/biostatgv/?module=tests>).

Results

Purification of zeaxanthin from *Porphyridium purpureum*

RP-HPLC of the ethanolic extract of *P. purpureum* gave a chromatogram containing eight major peaks at 436 nm (Fig. 2). The first pigment eluting as a major peak at 24.582 min was identified as zeaxanthin (**1**) as it presented a median polarity with maximal absorption wavelengths at 452 and 481 nm (Fig. 3), a III:II band ratio of 29.03% (Fig. 3), a high resolution molecular weight of 568.4294 (Fig. 4) and a MS^E fragmentation pattern characteristic of zeaxanthin (Fig. 4) (Juin et al., 2015).

Exposure to zeaxanthin inhibits the proliferation of A2058 melanoma cells

A 72 h exposure to increasing concentrations of zeaxanthin [0–60 μM] induced a dose-dependent reduction in the number of A2058 cells as compared to the untreated control, reaching 60% growth inhibition. The IC_{50} value of zeaxanthin was determined as $40 \mu\text{M}$ using the free Graph pad Prism software “sigmoidal dose response” (variable slope) function (Fig. 5).

Exposure to zeaxanthin evoked cytotoxicity and nuclear fragmentation in A2058 cells

A2058 cells incubated in control medium showed a regular epithelial shape, except for mitotic cells exhibiting a round shape and weak adhesion to the culture dish (Fig. 6A). The nucleus of

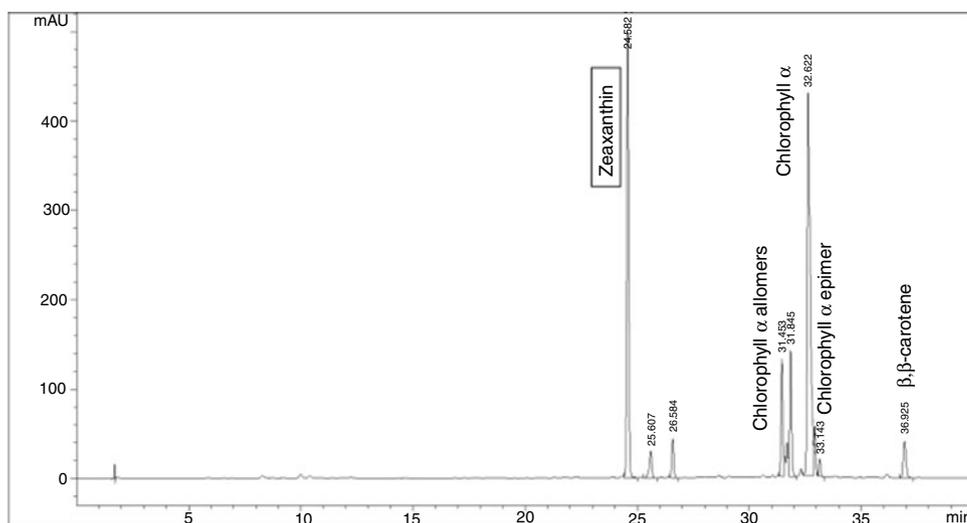


Fig. 2. RP-HPLC Chromatogram of *Porphyridium purpureum* ethanol extract at 436 nm obtained using the Van Heukelem and Thomas analysis (Van Heukelem and Thomas, 2001). The pigment profile was identical to that previously reported in Serive et al. (2017).

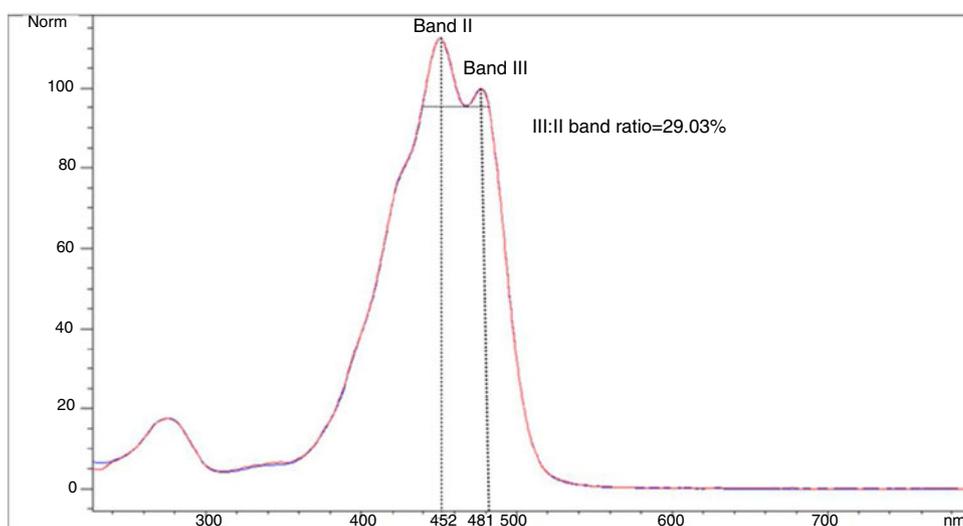


Fig. 3. Identification of the pigment eluting at 24.582 min as zeaxanthin (1) based on absorption spectrum, maximal absorbance wavelengths and III:II band ratio.

control cells was round and showed no sign of DNA condensation, blebbing or shrinkage (Fig. 6B). The treatment with staurosporine 2 μ M (non selective kinases inhibitor, control apoptosis inducer) evoked a high cytotoxicity evidenced by cell shrinkage (Fig. 6C) and DNA condensation in the nucleus (Fig. 6D). A 72 h treatment with zeaxanthin 40 μ M half lowered the cell density, evoked rounding of the cells (Fig. 6E) and nuclear fragmentation (Fig. 6F), suggesting a blockade in the cell cycle and apoptosis induction.

Zeaxanthin induces apoptosis in A2058 cells

To evaluate the effect of zeaxanthin on cell death, double fluorescence staining with Annexin V and 6-CFDA was performed to differentiate live and apoptotic cells. 6-CFDA is used to measure viability. In this sense, live cells will only stain with 6-CF (green), cells in early apoptosis will stain both with Annexin V (red) and 6-CF (green), and cells in late apoptosis will only stain with Annexin V and DAPI. After 72 h of treatment, zeaxanthin 40 μ M increased the number of Annexin V and 6-CFDA double-stained cells, and Annexin V and DAPI stained cells compared to control, indicating

enhancement of apoptosis (Fig. 7). A similar result was observed for staurosporine (1 μ M), a known pro-apoptotic agent.

Exposure to zeaxanthin evoked caspase-3 activation, internucleosomal fragmentation and hypopolyploidy in A2058 cells

Activation of caspase-3 is considered as a central event for integrating pro-apoptotic stimuli and activating downstream effector caspases and DNases during cancer cell apoptosis. The basal activity of caspase-3 was low in A2058 cells grown for 72 h in control cell culture medium (Fig. 8). Treatment with zeaxanthin 40 μ M for 72 h induced a very significant increase in caspase-3 activity (Student *t* test, $p < 0.01$), as demonstrated by hydrolysis of the specific chromogenic substrate Ac-DEVD-pNA (Fig. 8). Treatment with staurosporine 2 μ M, used as a positive control for caspase-3 activation, induced a highly significant increase in caspase-3 activity (Student *t* test, $p < 0.001$).

As one of the main consequences of caspase-3 activation is the subsequent DNA fragmentation by caspase-activated DNases, we evaluated the DNA content and DNA internucleosomal

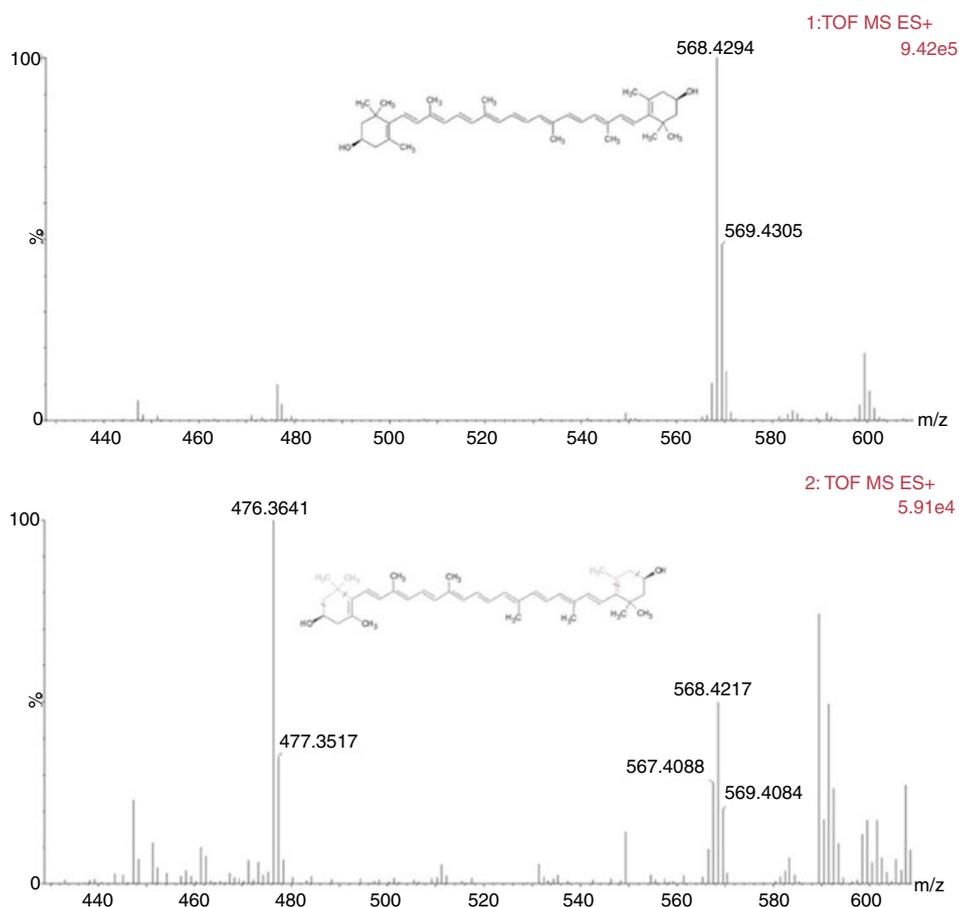


Fig. 4. MS^E fragmentation pattern of zeaxanthin (1) isolated from *Porphyridium purpureum*.

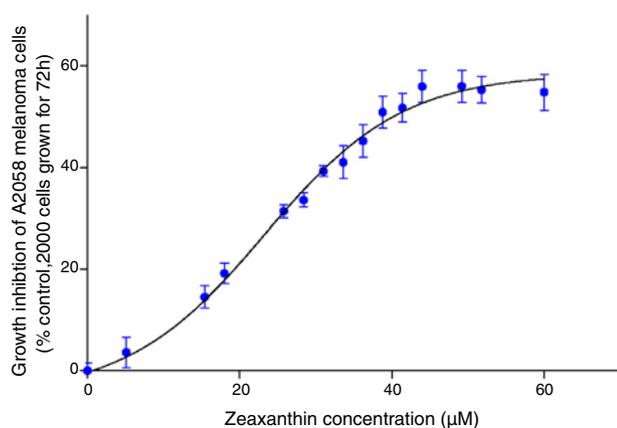


Fig. 5. Growth inhibition of A2058 cell in the presence of zeaxanthin (1). A2058 cells were grown for 72 h in a cell culture medium containing increasing concentrations of zeaxanthin. The antiproliferative activity of zeaxanthin was observed for concentrations superior to 5 μM and IC_{50} was determined as 40 μM .

fragmentation in A2058 cells treated with zeaxanthin. Evaluation of cell cycle progression by flow cytometry revealed the appearance of a sub-G1 cell population after exposure to zeaxanthin 40 μM , characteristic of dying cells (Fig. 9). Quantification using the flux cytometer software indicated that $2.9 \pm 0.4\%$ of control cells were in sub-G1 phase in comparison with $23.6 \pm 3.7\%$ in zeaxanthin-treated cells.

Agarose gel electrophoresis confirmed the internucleosomal fragmentation of DNA extracted from A2058 cells treated with

zeaxanthin 40 μM or staurosporine 2 μM (Fig. 10), demonstrating the activation of caspase-activated DNase by zeaxanthin.

Zeaxanthin stimulates the expression of the pro-apoptotic factors Bim and Bid and inhibits the nuclear translocation of NF- κB in A2058 melanoma cells

It was previously reported that zeaxanthin decreased the expression of antiapoptotic proteins (Bcl-2 and Bcl-xL) and increased the expression of proapoptotic proteins (Bak and Bax) in zeaxanthin-treated uveal melanoma cells (Bi et al., 2013). To complete the identification of signaling pathways involved in zeaxanthin-induced apoptosis of melanoma cells (Bi et al., 2013), the expression of pro-apoptotic, anti-apoptotic and pro-inflammatory factors were investigated by western-blot analysis. Zeaxanthin 40 μM respectively induced a high and moderate expression increase of the pro-apoptotic factors Bim and Bid (Fig. 11) that could be involved in the onset of apoptosis. The expression of the pro-apoptotic factor Bak was unchanged. The level of phosphorylated NF- κB p65 (Ser 536) was decreased while I $\kappa\text{B}\alpha$ and I $\kappa\text{B}\beta$ were up-regulated, indicating that zeaxanthin inhibited the nuclear translocation of NF- κB and subsequently down-regulated the expression of pro-inflammatory genes. I $\kappa\text{B}\beta$ and unphosphorylated NF- κB expressions were unchanged (Fig. 11).

Zeaxanthin potentiates the antiproliferative activity of Vemurafenib in A2058 melanoma cells

To assess the capacity of zeaxanthin to potentiate the growth inhibition induced by vemurafenib, a BRAF inhibitor used for the

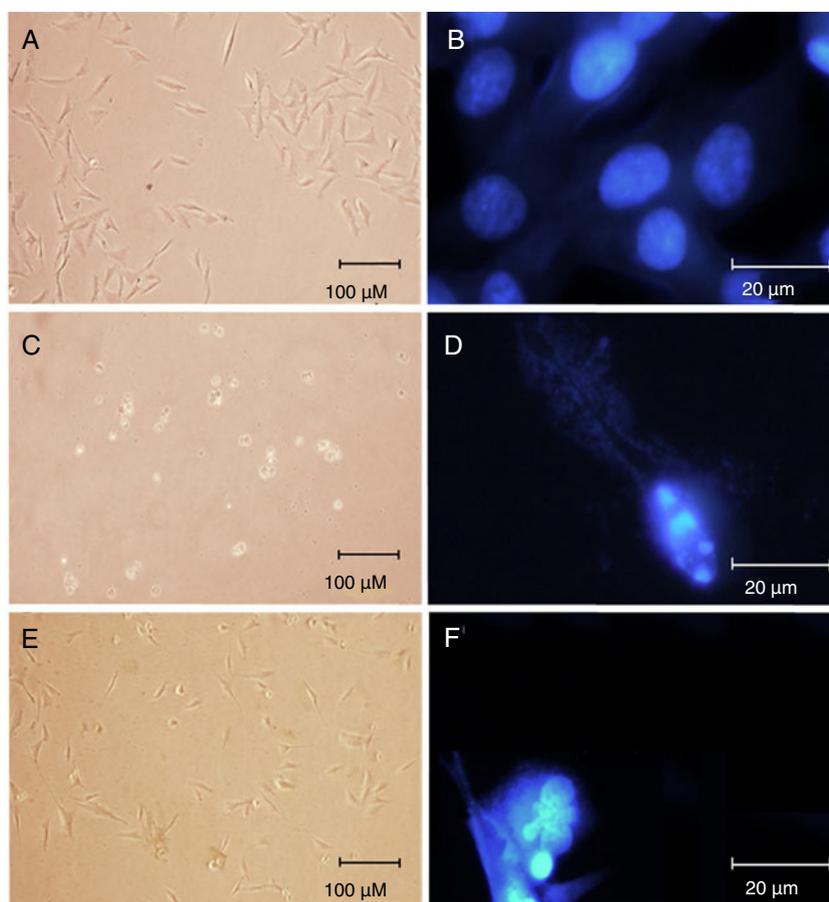


Fig. 6. Cytotoxicity and nuclear fragmentation in A2058 cells exposed to zeaxanthin 40 μM . A2058 cells were grown for 72 h in a control cell culture medium (A and B) or in a medium containing staurosporine 2 μM (C and D) or zeaxanthin 40 μM (E and F). Treatment with staurosporin evoked cell rounding and chromatin condensation (C and D) while zeaxanthin evoked cell rounding (E), chromatin condensation and nuclear fragmentation (F).

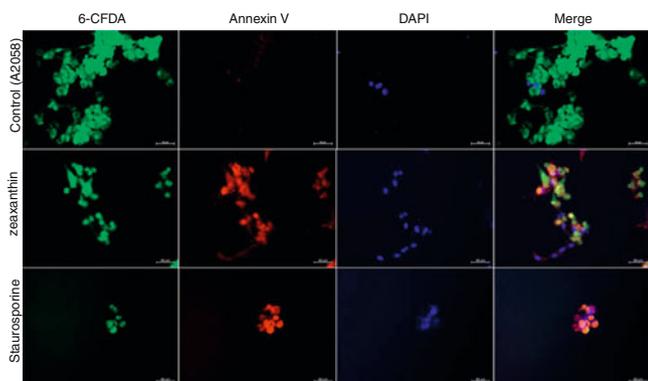


Fig. 7. Zeaxanthin induces apoptosis of A2058 melanoma cells. Annexin V (red) and 6-CFDA (green) double staining of apoptotic cells was examined by fluorescence microscopy. A2058 cells in early apoptosis showed both green and red stains; A2058 cells in late apoptosis showed red and blue stains; and control (untreated) cells stained green only. Cells treated with zeaxanthin (40 μM , for 72 h) or Staurosporine (1 μM , for 24 h) were considered in early or late apoptosis compared to control. Scale bar: 50 μm .

clinical treatment of metastatic melanoma, A2058 melanoma cells were treated for 72 h with zeaxanthin 40 μM , vemurafenib 5 μM or the combination of both molecules. Control A2058 cells exhibited a regular epithelial morphology and became sub-confluent in 72 h (Fig. 12A), with a high proportion of mitotic cells indicating a high proliferation rate. Zeaxanthin 40 μM induced a reduction in cell density, cell shrinkage, DNA condensation evidenced by the observation of nuclear granulations and appearance of apoptotic cells

(black arrows) (Fig. 12B). Vemurafenib 5 μM had a drastic effect on A2058 cell proliferation and morphology, evidenced by a low cell density, cell body shrinkage, cell thinning and DNA condensation with the presence of nuclear granulations (Fig. 12C). The morphology of cells treated with the combination of zeaxanthin and vemurafenib was similar to that observed with the vemurafenib treatment alone (Fig. 12D). As compared to the control cell culture medium, zeaxanthin 40 μM induced $44.1 \pm 6.4\%$ growth inhibition, vemurafenib 0.1 μM 10.40 ± 2.12 , 1 μM 32.74 ± 2.71 , and 5 μM $67.3 \pm 7.7\%$ growth inhibition and the combination of both 48.38 ± 3.98 , 58.48 ± 3.80 and $74.5 \pm 6.2\%$ growth inhibition, respectively. Thus zeaxanthin 40 μM induced a 37.98, 25.75 and 10.7% increase of the antiproliferative activity of vemurafenib 0.1, 1 and 5 μM , respectively (Fig. 12E). This value was in the range of calculated standard deviations of antiproliferative activities.

Discussion

Advanced melanoma have a bad prognosis as most molecules used in cancer chemotherapy are ineffective in killing metastatic melanoma cells which are constitutively or adaptatively resistant to pro-apoptotic drugs. The development of targeted therapies using BRAF inhibitors has significantly improved the treatment of metastatic melanomas as the V600E BRAF oncogenic mutation is found in more than 70% of clinical cases. However most patients eventually develop resistance mechanisms that ultimately lead to therapeutic impasses. In this view, many research projects aim to identify natural molecules with cytostatic, antimetastatic and anti-angiogenic activities that do not weaken the immune system

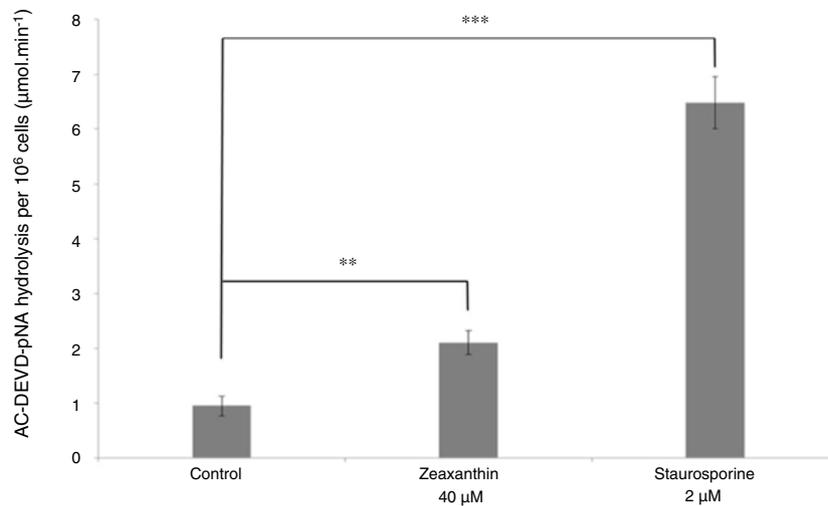


Fig. 8. Zeaxanthin induces caspase-3 activation in A2058 melanoma cells. A2058 cells were grown for 72 h in a control cell culture medium or in a medium containing zeaxanthin 40 μM or staurosporine 2 μM (positive control for apoptosis induction).

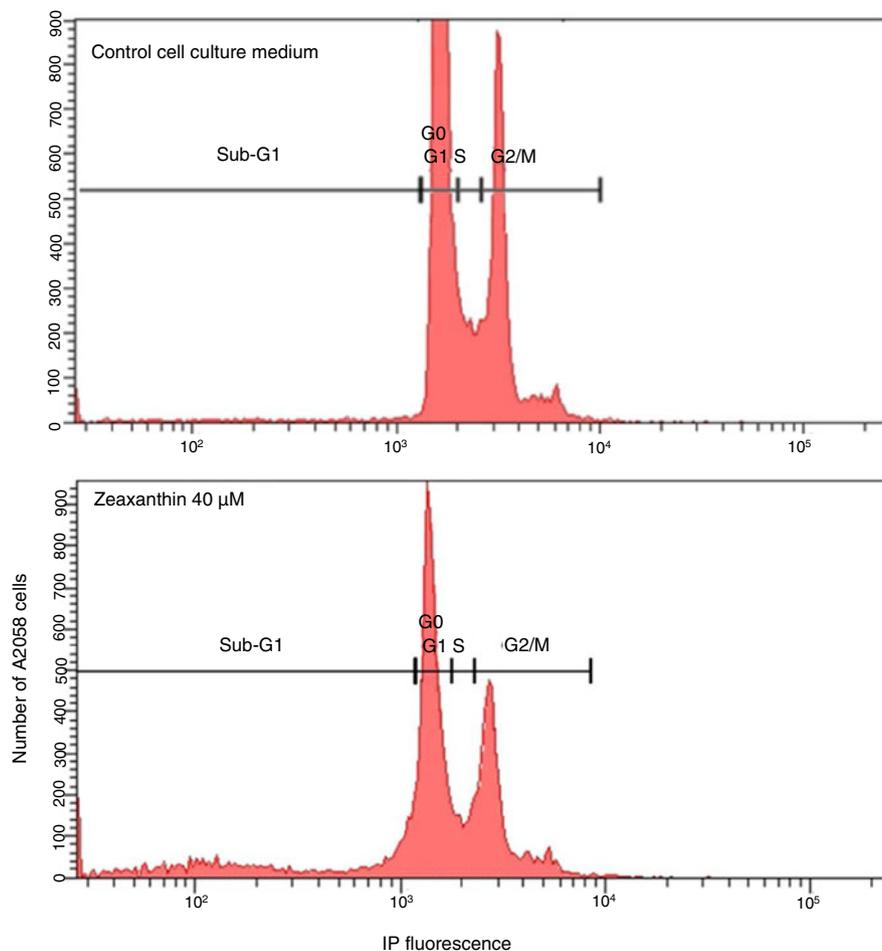


Fig. 9. Cell cycle analysis of A2058 cells grown for 72 h in control cell culture medium (A) or cell culture medium containing zeaxanthin 40 μM (B). The majority of control or treated cells were in the quiescence (G0) or pre-replicative (G1) phases. Zeaxanthin induced the appearance of a sub-G1 peak, characteristic of hypodiploid cells undergoing cell deaths.

and could be used to potentiate the efficiency of chemotherapy and immunotherapy and slow the emergence of resistance mechanisms (Cragg et al., 1997; Caltagirone et al., 2000; Niles et al., 2003; Mesquita et al., 2009; Van Goietsenoven et al., 2010; Pasquet et al., 2011; Ahmad et al., 2013; Zhang et al., 2014; Alqathama and

Prieto, 2015; Mirzaei et al., 2016). Many carotenoids meet all these activities, as they display high cytotoxicity in tumor cells from various histological origins, including chemoresistant melanoma cell lines, and exert significant antitumoral activity *in vivo* by inhibiting tumor growth, angiogenesis and invasivity (Gagez et al., 2012;

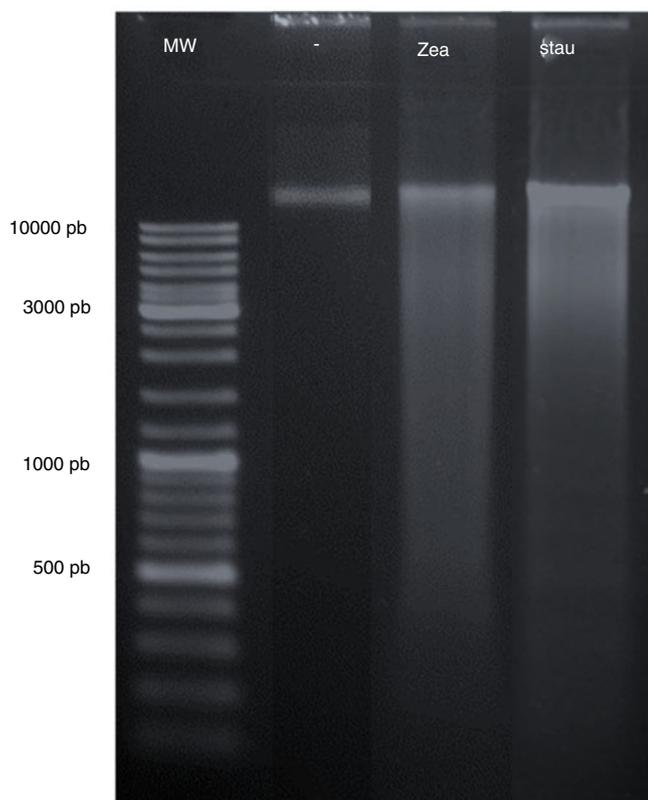


Fig. 10. Agarose gel electrophoresis of DNA extracted from A2058 cells incubated for 72 h in the absence (–) or presence of zeaxanthin 40 μ M (zea) or staurosporine 2 μ M (stau). Observation of a smear in the zeaxanthin and staurosporine lanes revealed the activation of caspase-activated DNases and internucleosomal fragmentation of DNA in apoptotic cells.

Kumar et al., 2013; Chen et al., 2017). Moreover, most carotenoids have no oral toxicity and do not weaken the immune system (Chew and Park, 2004; Pechinskii and Kuregyan, 2014; Ghodrati-zadeh et al., 2014). Microalgae constitute an optimal source to produce carotenoids for pharmaceutical applications as they combine the

advantages of synthesizing the wide chemodiversity of carotenoids, with high production yields, without the need of freshwater, agricultural surfaces or pesticides to be grown (Mimouni et al., 2012). Selection of hyper-producing strains combined to optimization of their growth conditions and purification processes allow the recovery of high amounts of carotenoids devoid of chemicals or endotoxins. In the present report, we demonstrate that zeaxanthin, an abundant carotenoid present in microalgae, that can be easily obtained in high amounts from the rhodophyte *P. purpureum*, induces apoptosis in human melanoma cells expressing the oncogenic BRAF V600E mutation and potentiates the antiproliferative activity of vemurafenib, a BRAF inhibitor used in patients with advanced metastatic melanomas. Zeaxanthin (1) was previously reported to have no oral toxicity and decrease the incidence of various cancers after oral ingestion (Thurnham and Howard, 2013; Xu et al., 2013). It was also reported to exert pro-apoptotic activity in human uveal melanoma cells (SP6.5 and C918) and limit uveal melanoma invasivity without impairing the viability of non cancer uveal melanocytes (Xu et al., 2015; Bi et al., 2013). In A2058 melanoma cells, zeaxanthin IC_{50} was determined as 40 μ M, a concentration inducing cell rounding, chromatin condensation, nuclear fragmentation, hypodiploidy, cell apoptosis in early and late stages, accumulation of cells in sub-G1 phase, DNA internucleosomal fragmentation and activation of caspase-3. Zeaxanthin-induced apoptosis was accompanied by inhibition of NF- κ B and up-regulation of the pro-apoptotic factors Bim and Bid, demonstrating the involvement of the mitochondrial signaling pathway in apoptosis triggering, as previously reported in uveal melanoma models (Bi et al., 2013). The observation that zeaxanthin was able to moderately potentiate the *in vitro* antiproliferative activity of vemurafenib is a promising result of our study as it suggests its potential interest as a nutritional adjuvant increasing the sensitivity of tumor cells to BRAF inhibitors. By potentiating the antiproliferative effect of BRAF inhibitors, zeaxanthin may allow to decrease BRAF inhibitors effective doses, limit their adverse effects in patients and delay the emergence of resistance mechanisms (Chu et al., 2012; Zimmer et al., 2012; Anforth et al., 2015; Welsh and Corrie, 2015). The molecular mechanisms involved in this increase of sensitivity as well as preclinical and clinical relevance of combining carotenoids with BRAF inhibitors will have to be further

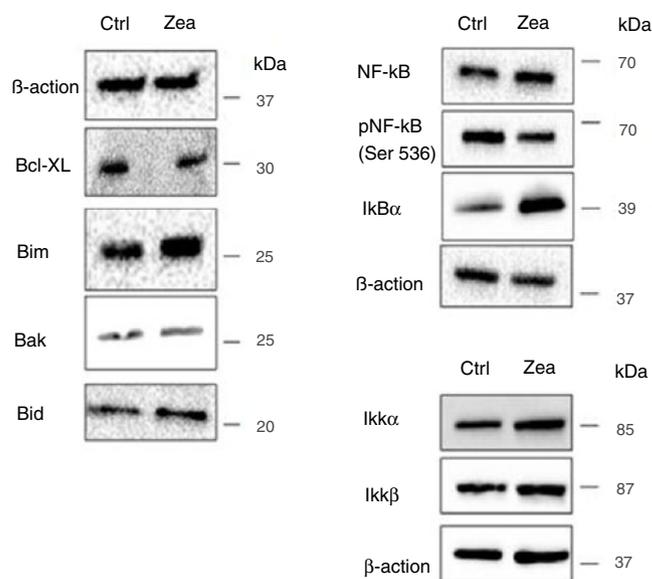


Fig. 11. Effect of zeaxanthin on the expression of pro-apoptotic and anti-apoptotic factors and activation of pro-inflammatory pathways in A2058 melanoma cells. Cells were incubated in control culture medium or in the presence of zeaxanthin 40 μ M for 72 h before protein extraction, SDS-PAGE and western blot.

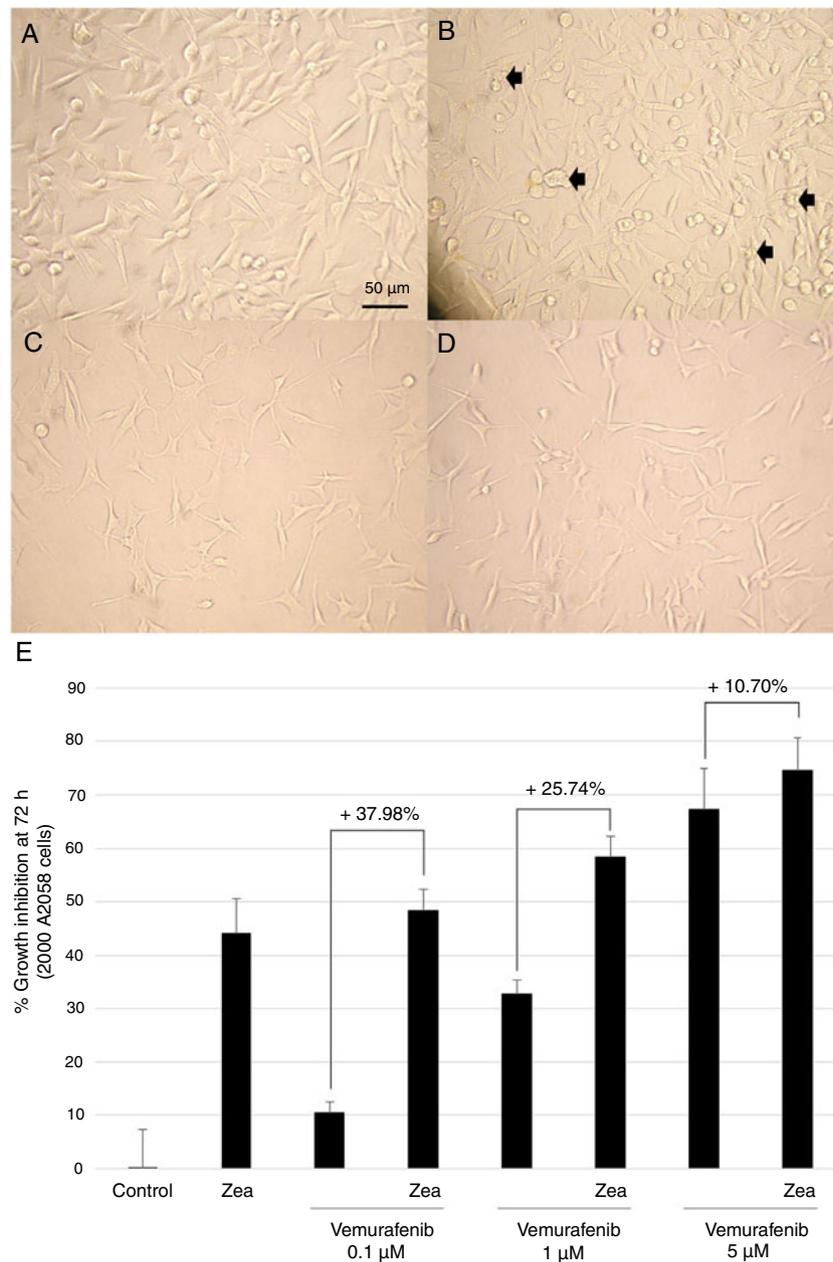


Fig. 12. Zeaxanthin sensitizes A2058 melanoma cells to the BRAF inhibitor vemurafenib. Two thousands A2058 melanoma cells were grown for 72 h in control cell culture medium (A), medium containing zeaxanthin 40 μM (B), vemurafenib 5 μM (C), or a mix of zeaxanthin 40 μM and vemurafenib 5 μM (D). Zeaxanthin induced a 37.98, 25.75 and 10.70% increase of the antiproliferative activity of vemurafenib 0.1, 1 and 5 μM, respectively (E).

explored, as this strategy could extend the duration of chemotherapy efficiency. In summary, this study confirms the potential of zeaxanthin to limit the growth of chemoresistant melanoma cells, confirms the major interest of phytoplankton carotenoids as natural anticancer molecules devoid of oral toxicity, and suggests for the first time the interest of combining a carotenoid to BRAF inhibitors to potentiate their chemotherapeutic efficiency in melanoma cells.

Authors' contribution and responsibility

JBB and EN produced *P. purpureum* biomass. CJ, RGOJ, AF, CO, LPy, IL, GP and LP performed the pigment extraction, purification, cell culture, western-blot and apoptosis experiments. CJ performed the HRMS analysis. LB and CJ performed the flux cytometry analysis. LP designed the experiments, interpreted the data, directed the study

and wrote the manuscript in collaboration with VT and JRGDSA. LP takes responsibility for the integrity of the work, from inception to finished article.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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References

- Ahmad, I., Muneer, K.M., Tamimi, I.A., Chang, M.E., Ata, M.O., Yusuf, N., 2013. Thymoquinone suppresses metastasis of melanoma cells by inhibition of NLRP3 inflammasome. *Toxicol. Appl. Pharmacol.* 270, 70–76.
- Alqathama, A., Prieto, J.M., 2015. Natural products with therapeutic potential in melanoma metastasis. *Nat. Prod. Rep.* 32, 1170–1182.
- Anforth, R., Carlos, G., Clements, A., Kefford, R., Fernandez-Peñas, P., 2015. Cutaneous adverse events in patients treated with BRAF inhibitor-based therapies for metastatic melanoma for longer than 52 weeks. *Br. J. Dermatol.* 172, 239–243.
- Armstrong, B.K., Kricger, A., 2001. The epidemiology of UV induced skin cancer. *J. Photochem. Photobiol. B* 63, 8–18.
- Baudelet, P.H., Gagez, A.L., Bérard, J.B., Juin, C., Bridiau, N., Kaas, R., Thiéry, V., Cadoret, J.P., Picot, L., 2013. Antiproliferative activity of *Cyanophora paradoxa* pigments in melanoma, breast and lung cancer cells. *Mar. Drugs* 11, 4390–4406.
- Bi, M.C., Rosen, R., Zha, R.Y., McCormick, S.A., Song, E., Hu, D.N., 2013. Zeaxanthin induces apoptosis in human uveal melanoma cells through Bcl-2 family proteins and intrinsic apoptosis pathway. *Evid. Based. Complement. Alternat. Med.*, <http://dx.doi.org/10.1155/2013/205082>.
- Burri, B.J., Neidlinger, T.R., Clifford, A.J., 2001. Serum carotenoid depletion follows first-order kinetics in healthy adult women fed naturally low carotenoid diets. *J. Nutr.* 131, 2096–2100.
- Caltagirone, S., Rossi, C., Poggi, A., Ranelletti, F.O., Natali, P.G., Brunetti, M., Aiello, F.B., Piantelli, M., 2000. Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *Int. J. Cancer* 87, 595–600.
- Chen, Y.T., Kao, C.J., Huang, H.Y., Huang, S.Y., Chen, C.Y., Lin, Y.S., Wen, Z.H., Wang, H.M.D., 2017. Astaxanthin reduces MMP expressions, suppresses cancer cell migrations, and triggers apoptotic caspases of in vitro and in vivo models in melanoma. *J. Funct. Foods* 31, 20–31.
- Chew, B.P., Park, J.S., 2004. Carotenoid action on the immune response. *J. Nutr.* 134, 257S–261S.
- Chu, E.Y., Wanat, K.A., Miller, C.J., Amaravadi, R.K., Fecher, L.A., Brose, M.S., McGettigan, S., Giles, L.R., Schuchter, L.M., Seykora, J.T., Rosenbach, M., 2012. Diverse cutaneous side effects associated with BRAF inhibitor therapy: a clinicopathologic study. *J. Am. Acad. Dermatol.* 67, 1265–1272.
- Chung, T.W., Choi, H.J., Lee, J.Y., Jeong, H.S., Kim, C.H., Joo, M., Choi, J.Y., Han, C.W., Kim, S.Y., Choi, J.S., Ha, K.T., 2013. Marine algal fucoxanthin inhibits the metastatic potential of cancer cells. *Biochem. Biophys. Res. Commun.* 439, 580–585.
- Cragg, G.M., Newman, D.J., Snader, K.M., 1997. Natural products in drug discovery and development. *J. Nat. Prod.* 60, 52–60.
- Dankort, D., Curley, D.P., Cartlidge, R.A., Nelson, B., Karnezis, A.N., Damsky, W.E., You, M.J., De Pinho, R.A., McMahon, M., Bosenberg, M., 2009. *Braf*(V600E) cooperates with *Pten* loss to induce metastatic melanoma. *Nat. Genet.* 41, 544–552.
- Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B.A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G.J., Bigner, D.D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J.W.C., Leung, S.Y., Yuen, S.T., Weber, B.L., Seigler, H.F., Darrow, T.L., Paterson, H., Marais, R., Marshall, C.J., Wooster, R., Stratton, M.R., Futreal, P.A., 2002. Mutations of the *BRAF* gene in human cancer. *Nature* 417, 949–954.
- Dutton-Regester, K., Irwin, D., Hunt, P., Aoude, L.G., Tembe, V., Pupo, G.M., Lanagan, C., Carter, C.D., O'Connor, L., O'Rourke, M., Scolyer, R.A., Mann, G.J., Schmidt, C.W., Herington, A., Hayward, N.K., 2012. A high-throughput panel for identifying clinically relevant mutation profiles in melanoma. *Mol. Cancer Ther.* 11, 888–897.
- Firdous, A.P., Sindhu, E.R., Ramnath, V., Kuttan, R., 2010. Anti-mutagenic and anti-carcinogenic potential of the carotenoid meso-zeaxanthin. *Asian Pac. J. Cancer Prev.* 11, 1795–1800.
- Gagez, A.L., Thiéry, V., Pasquet, V., Cadoret, J.P., Picot, L., 2012. Epoxycarotenoids and cancer. *Curr. Bioact. Compd.* 8, 109–141.
- Ghodratizadeh, S., Kanbak, G., Beyramzadeh, M., Dikmen, Z.G., Memarzadeh, S., Habibi, R., 2014. Effect of carotenoid β -cryptoxanthin on cellular and humoral immune response in rabbit. *Vet. Res. Commun.* 38, 59–62.
- Grange, F., 2005. Epidemiology of cutaneous melanoma: descriptive data in France and Europe. *Ann. Dermatol. Venerol.* 132, 975–982.
- Hall, W.A., Djaliliani, H.R., Nussbaum, E.S., Cho, K.H., 2000. Long-term survival with metastatic cancer to the brain. *Med. Oncol.* 17, 279–286.
- Haluska, F.G., Tsao, H., Wu, H., Haluska, F.S., Lazar, A., Goel, V., 2006. Genetic alterations in signaling pathways in melanoma. *Clin. Cancer Res.* 12, 2301s–2307s.
- Hashimoto, T., Ozaki, Y., Mizuno, M., Yoshida, M., Nishitani, Y., Azuma, T., Komoto, A., Maoka, T., Tanino, Y., Kanazawa, K., 2011. Pharmacokinetics of fucoxanthin in human plasma after the oral administration of kombu extract. *Br. J. Nutr.* 107, 1–4.
- Hedidi, M., Erb, W., Bentabed-Ababsa, G., Chevallier, F., Picot, L., Thiéry, V., Bach, S., Ruchaud, S., Roisnel, T., Dorcet, V., Mongin, F., 2016. Synthesis of *N*-pyridyl azoles using a deprotonation-iodolysis-*N*-arylation sequence and evaluation of their antiproliferative activity in melanoma cells. *Tetrahedron* 72, 6467–6476.
- Jang, S., Atkins, M.B., 2014. Treatment of BRAF-mutant melanoma: the role of vemurafenib and other therapies. *Clin. Pharmacol. Ther.* 95, 24–31.
- Juin, C., Bonnet, A., Nicolau, E., Bérard, J.B., Devillers, R., Thiéry, V., Cadoret, J.P., Picot, L., 2015. UPLC-MSE profiling of phytoplankton metabolites: application to the identification of pigments and structural analysis of metabolites in *Porphyridium purpureum*. *Mar. Drugs* 13, 2541–2558.
- Kim, K.N., Ahn, G., Heo, S.J., Kang, S.M., Kang, M.C., Yang, H.M., Kim, D., Roh, S.W., Kim, S.K., Jeon, B.T., Park, P.J., Jung, W.K., Jeon, Y.J., 2013. Inhibition of tumor growth in vitro and in vivo by fucoxanthin against melanoma B16F10 cells. *Environ. Toxicol. Pharmacol.* 35, 39–46.
- Kumar, S.R., Hosokawa, M., Miyashita, K., 2013. Fucoxanthin: a marine carotenoid exerting anti-cancer effects by affecting multiple mechanisms. *Mar. Drugs* 11, 5130–5147.
- Leiter, U., Garbe, C., 2008. Epidemiology of melanoma and nonmelanoma skin cancer – the role of sunlight. In: *Sunlight, Vitam. D Ski. Cancer*. Springer, New York, NY, pp. 89–103.
- Locatelli, C., Filippin-Monteiro, F.B., Creczynski-Pasa, T.B., 2013. Recent advances in the biology, therapy and management of melanoma. *Int. Tech.*
- Lu, M., Zhang, Y., Zhao, C., Zhou, P., Yu, L., 2015. Analysis and identification of astaxanthin and its carotenoid precursors from *Xanthophyllomyces dendrorhous* by high-performance liquid chromatography. *Z. Naturforsch. C* 65, 489–494.
- MacKie, R.M., Hauschild, A., Eggermont, A.M.M., 2009. Epidemiology of invasive cutaneous melanoma. *Ann. Oncol.* 20 (Suppl. 6 vi), 1–7.
- Marneros, A.G., 2009. Tumor angiogenesis in melanoma. *Hematol. Oncol. Clin. North Am.* 23, 431–446.
- Mathieu, V., de Lassalle, E.M., Toelen, J., Mohr, T., Bellahcène, A., Van Goietsenoven, G., Verschuer, T., Bouzin, C., Debeyer, Z., De Vleeschouwer, S., Van Gool, S., Poirier, F., Castronovo, V., Kiss, R., Feron, O., 2012. Galectin-1 in melanoma biology and related neo-angiogenesis processes. *J. Invest. Dermatol.* 132, 2245–2254.
- Mesquita, M.L., Paula, J.E., Pessoa, C., Moraes, M.O., Costa-Lotufo, L.V., Grougnet, R., Michel, S., Tillequin, F., Espindola, L.S., 2009. Cytotoxic activity of Brazilian *Cerrado* plants used in traditional medicine against cancer cell lines. *J. Ethnopharmacol.* 123, 439–445.
- Mimouni, V., Ulmann, L., Pasquet, V., Mathieu, M., Picot, L., Bougaran, G., Cadoret, J.P., Morant-Manceau, A., Schoefs, B., 2012. The potential of microalgae for the production of bioactive molecules of pharmaceutical interest. *Curr. Pharm. Biotechnol.* 13, 2733–2750.
- Mirzaei, H., Naseri, G., Rezaee, R., Mohammadi, M., Banikazemi, Z., Mirzaei, H.R., Salehi, H., Peyvandi, M., Pawelek, J.M., Sahebkar, A., 2016. Curcumin: a new candidate for melanoma therapy? *Int. J. Cancer* 139, 1683–1695.
- Niles, R.M., McFarland, M., Weimer, M.B., Redkar, A., Fu, Y.M., Meadows, G.G., 2003. Resveratrol is a potent inducer of apoptosis in human melanoma cells. *Cancer Lett.* 190, 157–163.
- Oliveira-Junior, R.G., Thiéry, V., Sergent, O., Chevanne, M., Picot, L., 2016. Could fucoxanthin interaction with lipid rafts mediate its cytotoxicity in cancer cells? *J. Oceanogr. Mar. Res.* 4, 144.
- Pasquet, V., Morisset, P., Ihammouine, S., Chépied, A., Aumailley, L., Berard, J.B., Serive, B., Kaas, R., Lanneluc, I., Thiéry, V., Lafferrère, M., Piot, J.M., Patrice, T., Cadoret, J.P., Picot, L., 2011. Antiproliferative activity of violaxanthin isolated from bio-guided fractionation of *Dunaliella tertiolecta* extracts. *Mar. Drugs* 9, 819–831.
- Pechinskii, S.V., Kuregyan, A.G., 2014. The impact of carotenoids on immunity (Review). *Pharm. Chem. J.* 47, 509–513.
- Putey, A., Joucla, L., Picot, L., Besson, T., Joseph, B., 2007. Synthesis of *latonduine* derivatives via intramolecular Heck reaction. *Tetrahedron* 63, 867–879.
- Reboul, E., 2013. Absorption of vitamin A and carotenoids by the enterocyte: focus on transport proteins. *Nutrients* 5, 3563–3581.
- Roy, S., Llewellyn, C., Egeland, E., Johnsen, G. (Eds.), 2011. *Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography* (Cambridge Environmental Chemistry Series). Cambridge University Press, Cambridge, pp. 728–822, <http://dx.doi.org/10.1017/CBO9780511732263.032>.
- Serive, B., Kaas, R., Bérard, J.B., Pasquet, V., Picot, L., Cadoret, J.P., 2012. Selection and optimisation of a method for efficient metabolites extraction from microalgae. *Bioresour. Technol.* 124, 311–320.
- Serive, B., Nicolau, E., Bérard, J.B., Kaas, R., Pasquet, V., Picot, L., Cadoret, J.P., 2017. Community analysis of pigment patterns from 37 microalgae strains reveals

- new carotenoids and porphyrins characteristic of distinct strains and taxonomic groups. PLOS ONE 12, e0171872.
- Spagnolo, F., Ghorzo, P., Queirolo, P., 2014. Overcoming resistance to BRAF inhibition in BRAF-mutated metastatic melanoma. *Oncotarget* 5, 10206–10221.
- Sugawara, T., Matsubara, K., Akagi, R., Mori, M., Hirata, T., 2006. Antiangiogenic activity of brown algae fucoxanthin and its deacetylated product, fucoxanthinol. *J. Agric. Food Chem.* 54, 9805–9810.
- Tanaka, T., Shnimitzu, M., Moriwaki, H., 2012. Cancer chemoprevention by carotenoids. *Molecules* 17, 3202–3242.
- Thurnham, D.I., Howard, A.N., 2013. Studies on meso-zeaxanthin for potential toxicity and mutagenicity. *Food Chem. Toxicol.* 59, 455–463.
- Van Goietsenoven, G., Hutton, J., Becker, J.P., Lallemand, B., Robert, F., Lefranc, F., Pirker, C., Vandenbussche, G., Van Antwerpen, P., Evidente, A., Berger, W., Prévost, M., Pelletier, J., Kiss, R., Kinzy, T.G., Kornienko, A., Mathieu, V., 2010. Targeting of eEF1A with Amaryllidaceae isocarboxtyrils as a strategy to combat melanomas. *FASEB J.* 24, 4575–4584.
- Van Heukelem, L., Thomas, C., 2001. Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chromatogr. A* 910, 31–49.
- Walne, P., 1966. Experiments in the large-scale culture of the larvae of *Ostrea edulis* (L.). In: *Fish. Investig. Ser. II. London Her Majesty's Station. Off.*, pp. iii. 53.
- Welsh, S.J., Corrie, P.G., 2015. Management of BRAF and MEK inhibitor toxicities in patients with metastatic melanoma. *Ther. Adv. Med. Oncol.* 7, 122–136.
- Xu, X., Zhang, L., Shao, B., Sun, X., Ho, C.T., Li, S., 2013. Safety evaluation of meso-zeaxanthin. *Food Control* 32, 678–686.
- Xu, X.L., Hu, D.N., Iacob, C., Jordan, A., Gandhi, S., Gierhart, D.L., Rosen, R., 2015. Effects of zeaxanthin on growth and invasion of human uveal melanoma in nude mouse model. *J. Ophthalmol.*, <http://dx.doi.org/10.1155/2015/392305>.
- Zapata, M., Rodriguez, F., Garrido, J., 2000. Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. *Mar. Ecol. Prog. Ser.* 195, 29–45.
- Zhang, L., Wei, Y., Zhang, J., 2014. Novel mechanisms of anticancer activities of green tea component epigallocatechin-3-gallate. *Anticancer Agents Med. Chem.* 14, 779–786.
- Zimmer, L., Livingstone, E.U., Hillen, S., Domkes, A., Becker, D., Schadendorf, D., 2012. Panniculitis with arthralgia in patients with melanoma treated with selective BRAF inhibitors and its management. *Arch. Dermatol.* 148, 357–361.