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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.



## Introduction

Melanomas account for less than 2% of skin cancers but are responsible for 80% of their mortality (Armstrong and Kricker, 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 oncogen 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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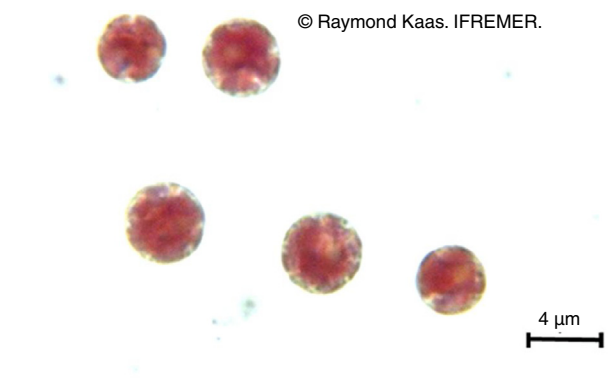



**Fig. 1.** *Porphyridium purpureum* strain CCAP 1380.3. © Raymond Kaas. IFREMER.

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-aptoptotic 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).

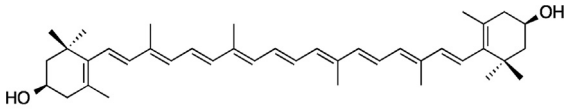
## Material and methods

### *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 µmol $m^{-2}$ $s^{-1}$ 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% $CO_2$ (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 mn, 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.




**1**

### *Cell culture*

A2058 (ATCC® CRL-11147™, 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% $CO_2$–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 µg $ml^{-1}$.

### *Determination of zeaxanthin $IC_{50}$ 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). $IC_{50}$ 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 $2 \times 10^5$ cells were seeded in 6-well plates, in a final volume of 3 ml of control medium or culture medium containing zeaxanthin 40 µM ($IC_{50}$) 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 µg $ml^{-1}$ 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.

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