SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

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TIPOLOGIA DI BORSA RICEVUTA: _____BORSA DI STUDIO PER SEI MESI_____

TIPOLOGIA DI RELAZIONE (es.: metà periodo o finale): _____RALEZIONE FINALE_____

TITOLO DELLA RELAZIONE:

Pro-apoptotic and anti-tumoral activities of natural compounds in melanoma

RELAZIONE:

Background

Melanoma is the most common form of skin cancer and it is responsible for the majority of skin cancer deaths. While early-stages melanoma can usually be effectively treated with surgery, more advanced tumors have a high metastatic potential and are notoriously resistant to conventional cancer therapies such as radiation and chemotherapy. Despite significant advances in understanding of melanoma biology and pathogenesis, and the recent success in developing targeted therapies for melanoma (Shtivelman E. et al. 2014, Tsao H. et al. 2012), the prognosis of the disease remains poor, therefore the search for new agents for its treatment is of great importance. The role of diet and nutrition in cancer prevention has recently become a very popular subject (Chen et al. 2014). Natural compounds and supplements have become part of people's daily life. Even though nutrients might never be as effective as chemotherapy or other pharmaceutical agents, their potential is clear, and may

also be effective as adjuvants to pharmaceutical drugs. Indicaxanthin, a phytochemical with the chemical structure of betalamic acid, is a dietary pigment from cactus pear fruit (*Opuntia ficus-indica, L. Miller*). In the first part of this project, *in vitro* studies showed that indicaxanthin inhibits human melanoma cell proliferation in a dose-dependent manner. The same experiment was carried out on murine melanoma cells B16/F10. In particular, incubation of A375 cells with indicaxanthin 50μM, 100μM and 200μM for 72h caused an inhibition of cell proliferation by 20,7%, 35,7% and 56%, respectively ($P < 0.001$); Instead, incubation of B16/F10 cells with indicaxanthin at the same doses for 72h caused an inhibition of cell proliferation by 33,8%, 46% and 69,1%, respectively ($P < 0.001$) (table present in the mid-term report). Moreover, cytofluorimetric analysis with annexin V/PI staining demonstrated that the anti-proliferative effect of indicaxanthin was due to its ability to induce apoptosis in A375 cells. In fact, previous experiments have shown that indicaxanthin induced apoptosis of A375 cell in a time-dependent manner (12% and 18% at 24 and 48h respectively) (figure present in the mid-term report). The apoptotic machinery can be controlled, at least in part, by NF-κB (Ben-Neriah and Karin, 2011). Several reports have shown that in melanoma the constitutive activation of NF-κB confers tumor survival capacity and avoidance of apoptosis (Ueda and Richmond, 2006). Thus, in the second part of this project, we have hypothesized that the indicaxanthin induction of apoptosis was associated with suppression of NF-κB activation. To further support our *in vitro* findings we also used indicaxanthin *in vivo* in a murine model of melanoma.

Methods

Electrophoretic mobility shift assay (EMSA)

Aliquots of total extracts (12lg protein/sample) in 0.1% Triton X-100 lysis buffer were incubated with ^{32}P-labeled κB DNA probes in binding buffer for 30min, as previously described (Panza et al., 2011). DNA-protein complexes were analyzed using non-denaturing 4% polyacrylamide gel electrophoresis. Quantitative evaluation of NF-κB-κB complex formation was done using a Typhoon-8600imager (Molecular Dynamics Phoshor-Imager, MDP, Amersham Biosciences, Piscataway, NJ, USA) and IMAGEQUANT software (Amer-sham Biosciences) (MDP analysis). For control of equal loading, NF-κB values were normalized to the level of the non-specific protein-DNA complex in the same lane.

In vivo 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. Female C57BL/6 mice (18-20g) were from Charles River Laboratories, Inc.

Induction of subcutaneous B16 lesions

Mice were subcutaneously (s.c.) injected in the right flank with B16-F10 cells ($1x10^5$/0.1 ml). When tumors reached an average diameter of 24mm, indicaxanthin (3,2mg/kg) was given orally three times a day, after 6 hours. 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(D1 x D2 x D3) where D1=length; D2=width; D3=height and expressed as cm^3.

Statistical analysis

Data from all *in vivo* experiments are reported as mean ± SEM unless otherwise noted. Data were analyzed 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 labeled with a single asterisk; P values less than 0.01 and 0.001 are designated with double and triple asterisks, respectively.

Results

Indicaxanthin inhibit NF-κB activation

Nuclear factor-κB (NF-κB) proteins are normally sequestered in the cytoplasm in an inactive form closely associated with the inhibitory protein inhibitor of kappa light chain gene enhancer in B cells-alpha (IκBa). To investigate the effect of indicaxanthin on NF-κB activity, A375 cells were treated with the compound (100μM) at different time points (1, 2, 4 and 8 h; Figure 2). The A375 cell line was found to display a constitutively high NF-κB DNA binding activity, which was reduced in a time-dependent manner by treatment with indicaxanthin.

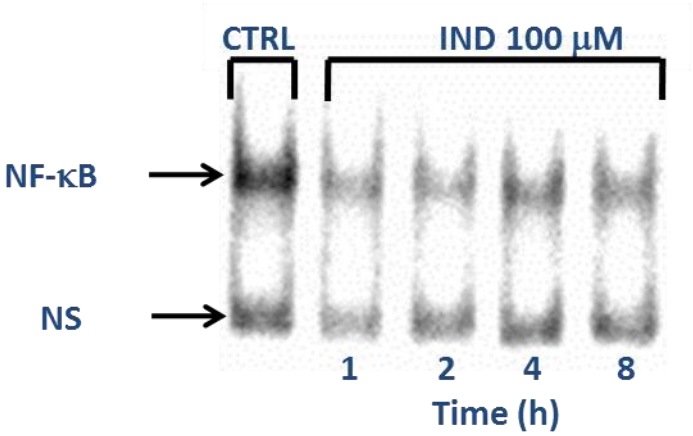

Figure 2: Nuclear extracts from control-treated and indicaxanthin-treated A375 cells collected at 1, 2, 4 and 8 h were analyzed by EMSA for NF-κB activation.

Indicaxanthin inhibits melanoma tumor in vivo in mice

To better elucidate the role of indicaxanthin in melanoma development and progression, we used a well known murine model of melanoma (Berkelhammer et al., 1982), which is induced by subcutaneously injecting B16-F10 murine cells in C57BL/6 mice. Indicaxanthin (3,2mg/kg) was administered orally to mice three times a day, after 6 hours. At day 14 after tumor implantation, a 69% reduction in tumor volume was observed in indicaxanthin-treated mice (0.064 ± 0.01 cm^3 mean tumor volume versus control mice 0.208 ± 0.002 cm^3 mean tumor volume, $P < 0.001$) (Figure 3).

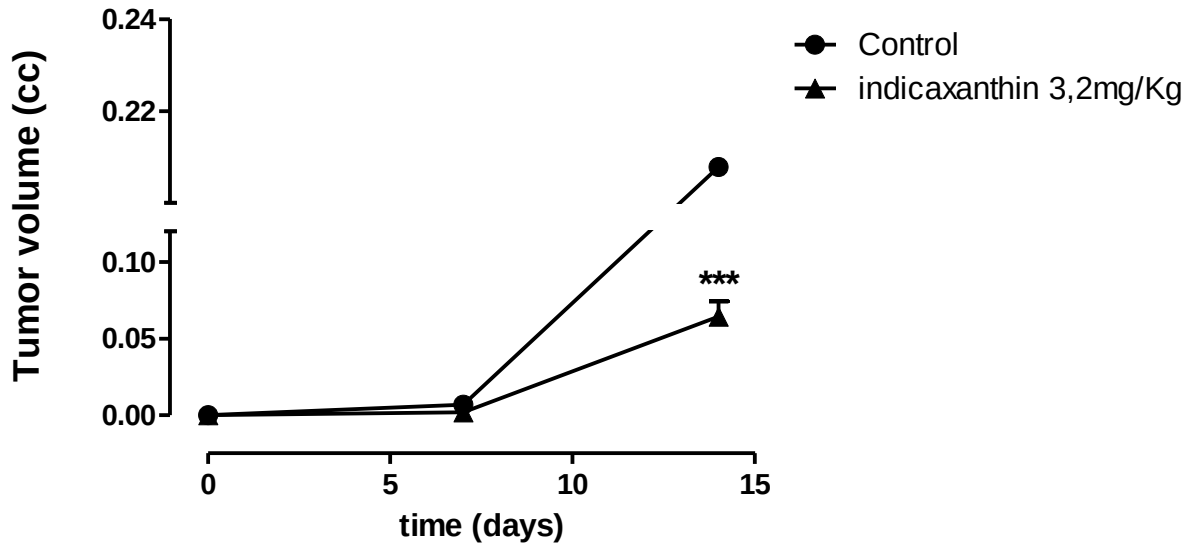

Figure 3: Indicaxanthin inhibits tumor growth in vivo.

Conclusion

We have demonstrated that indicaxanthin inhibits human melanoma cells proliferation by inhibiting pro-survival pathways associated to NF-κB activation. Moreover, indicaxanthin inhibits tumor growth in vivo. Our results establish this compound as new potential agents in the treatment of human melanoma and represent a very promising strategy to improve the fight against cancer.

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