β-CRYPTOXANTHIN SYNERGISTICALLY ENHANCES THE ANTITUMORAL ACTIVITY OF OXALIPLATIN THROUGH ΔNP73 NEGATIVE REGULATION IN COLON CANCER

Coral San Millán, Beatriz Soldevilla, Paloma Martín, Beatriz Gil-Calderón, Marta Compte, Belén Pérez-Sacristán, Encarnación Donoso, Cristina Peña, Jesús Romero, Fernando Granado-Lorencio, Félix Bonilla, and Gemma Domínguez

1Departamento de Medicina, Facultad de Medicina, Instituto de Investigaciones Biomédicas “Alberto Sols”, CSIC-UAM, 2Servicio de Anatomía Patológica, 3Grupo de señalización celular en cáncer, 4Unidad de inmunología molecular, 5Unidad de Vitaminas, 6Servicio de Bioquímica Clínica, 7Servicio de Oncología radioterápica, Hospital Universitario Puerta de Hierro Majadahonda, IDIPHIM, and 8Centro de Estudios Biosanitarios, Madrid, Spain.

*Correspondance to: Instituto de Investigaciones Biomédicas “Alberto Sols”, CSIC-UAM, C/ Arturo Duperier, 4, 28029, Madrid, Spain E-mail: gemma.dominguez@uam.es, felixbonillavelasco@gmail.com

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SIGNIFICANCE STATEMENT

Acquired resistance to chemotherapy is the major barrier in the treatment of many cancers. Thus, the development of new therapeutic approaches to overcome this limitation and improve patients’ outcomes is urgently needed. The addition of new compounds that negatively regulate genes involved in chemoresistance processes may help to overcome this obstacle. Here, we demonstrate that the natural carotenoid β-cryptoxanthin negatively regulates ΔNp73, an oncogenic variant of TP73 gene involved in drug resistance processes. Importantly, we propose a novel therapeutic strategy for the treatment of colon cancer based on the combination of β-cryptoxanthin with a conventional chemotherapeutic drug, oxaliplatin. The combined regimen produced more benefit than either individual treatment, without increasing side effects. In addition, the concentration-limiting toxicity of oxaliplatin could be reduced in the presence of the carotenoid.
ABSTRACT

BACKGROUND: The acquired resistance to chemotherapy represents the major limitation in the treatment of cancer. New strategies to solve this failure and improve patients’ outcomes are necessary. The cancer preventive effect of β-cryptoxanthin has been widely described in population studies. Few reports support its putative use as an antitumoral compound. Here we focus on the therapeutic potential of β-cryptoxanthin individually or in combination with oxaliplatin in colon cancer and try to decipher the molecular basis underlying its effect.

METHODS: Apoptosis, viability and proliferation assays, mice models and an intervention study in 20 healthy subjects were performed. A PCR array was carried out to unravel the molecular putative basis of the β-cryptoxanthin effect and further signaling experiments were conducted. Comet Assay was completed to evaluate the genotoxicity of the treatments.

RESULTS: β-cryptoxanthin differentially regulates the expression of the P73 variants in vitro, in vivo and in a human intervention study. This carotenoid decreases the proliferation of cancer cells and cooperates with oxaliplatin to induce apoptosis through the negative regulation of ΔNP73. The antitumoral concentrations of oxaliplatin decrease in the presence of β-cryptoxanthin to achieve same percentage of growth inhibition. The genotoxicity in PBMCs of mice decreased in the combined treatment.

CONCLUSIONS: We propose a putative novel therapeutic strategy for the treatment of colon cancer based on the combination of β-cryptoxanthin and oxaliplatin. The combined regimen produced more benefit that either individual modality without increasing side effects. Additionally, the concentration-limiting toxicity of oxaliplatin is reduced in the presence of the carotenoid.
INTRODUCTION

Diet certainly plays an important role in preventing cancer, but the underlying mechanism is still not clear. Epidemiological evidence links consumption of carotenoids with decreased risk of various types of cancer (1–4). β-cryptoxanthin is one of the six major carotenoids routinely measured in human serum. It is contained primarily in citrus fruits (5,6), but also found in corn, peas and some yellow animal products.

Numerous prospective studies show that serum levels of β-cryptoxanthin are inversely associated with the risk of a variety of cancers in several tissues (3, 7-19). However, controversial results have been published, such as those describing no association between β-cryptoxanthin and risk of colon (20), breast (21), ovarian (22) and prostate cancer (23) and non-Hodgkin lymphoma (24).

Additionally, several studies have demonstrated the protective effect of β-cryptoxanthin in carcinogenic-induced tumors of various tissues in animal models (4,25–28). In contrast, few studies examine its possible therapeutic potential. Thus, in vitro, β-cryptoxanthin suppresses the growth of immortalized human bronchial epithelial cells and non-small-cell lung cancer cells (29), stomach tumor cells (30) and melanoma(31). The mechanism by which β-cryptoxanthin exerts these effects remains largely unexplored (1). These findings nevertheless indicate that β-cryptoxanthin could be used as an antitumor compound.

Oxaliplatin, a third-generation platinum-based chemotherapy drug (32), has a major impact on the management and outcome of colorectal cancer patients (33) in combination with 5-FU and leucovorin (the FOLFOX regimen) or capcitabine (CapeOx regimen) for the adjuvant treatment of stage III (34). Other combined treatments are also in use in stage IV (35). However, the major obstacle in treating
colorectal cancer is still acquired resistance. The addition of new compounds to these treatments may overcome this obstacle and improve upon the outcomes currently achieved. In addition, new strategies that can diminish the neurotoxic effects of such treatments would be of interest (33). Our group has demonstrated the involvement of ΔNP73, an oncogenic variant of the TP73 gene, in proliferative and oxaliplatin chemotherapy resistance processes. Its overexpression is associated with lower survival rates in colon cancer patients (32), which points to a putative role as a therapeutic target.

In the present study β-cryptoxanthin in combination with oxaliplatin significantly enhanced apoptosis of colon cancer cells in vitro and in vivo. The combined treatment led to similar growth inhibition to those consisting of double individual oxaliplatin doses, which could eventually reduce the effective dose of oxaliplatin and therefore, the associated side effects. Accordingly, the Comet Assay showed a reduction of the genomic damage in healthy cells from those mice treated with the combined regimen. Finally, we identified the down-regulation of ΔNP73 variant as the mechanism underlying these effects. A human intervention study with a β-cryptoxanthin-enriched juice supports the in vivo regulation of P73 variants by this carotenoid.
MATERIALS AND METHODS

Cell culture

Human colon cancer cell lines HCT116 and SW1417 were obtained from the American Type Cell Collection, SW480-ADH cells are a subpopulation from SW480 (Aguilera et al., 2007) and the isogenic HCT116 TP53 null cells were kindly provided by C. Marín (Instituto de Biomedicina, Universidad de León, Spain). All cells were authenticated at the Genomic Service of our institution by the StemElite ID System (Promega). Cells were maintained as monolayer cultures in DMEM or RPMI (Lonza group Ltd, Switzerland) supplemented with 10% fetal bovine serum, 4 mmol/L L-glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Since, in our hands, HCT116 tumorigenicity in nude mice resulted higher than that from the other two cell lines, stable experiments were performed in this cell line in vitro. Thus, HCT116 cells were transiently or stably transfected with a pcDNA or pEGFP-1 plasmid encoding ΔNP73β or mock transfected (kindly provided by C. Marín, Instituto de Biomedicina, Universidad de León). One week post-transfection, pEGFP-1 expressing cells were selected by sorting and expanded in DMEM supplemented with 20% FBS.

Preparation of β-cryptoxanthin solution

Stock solution of β-cryptoxanthin (5 mmol/L) was prepared by dissolving β-cryptoxanthin (Extrasynthese, Lyon, France) in tetrahydrofuran (THF) and stored at -20°C until use. The trans-isomer of β-cryptoxanthin has been used throughout the study.

β-cryptoxanthin treatment
Aliquots from the stock solution were added to the cell culture medium at the desired working concentration. The final THF concentration in cell culture medium was 0.2% v/v. Control culture received only THF. Cells were plated on 6-well plates (2x10^5 cells/well) and grown overnight to allow for cell attachment. Cells were then treated with medium supplemented with β-cryptoxanthin or vehicle (0.2% THF v/v) for several periods of time. Cell culture medium was changed every 24 hours. All procedures were performed in the dark.

**Intervention study and lymphocyte extraction**

Twenty anonymous healthy volunteers were selected by non-probabilistic sampling. All participants were required to have biochemical and haematological profiles and serum levels of vitamin A, E and carotenoids within accepted reference ranges (5). The use of vitamin and/or herbal supplements, dieting, pregnancy, chronic medication or intercurrent disease or infection that could alter the bioavailability or status of the compounds of interest was used as exclusion criteria.

A juice containing 0.75 mg of β-cryptoxanthin was given to the healthy donors daily for one month. Although no side effects were expected from the intervention, subjects were additionally tested for general indexes in routine clinical practice.

The study protocol was approved by the Ethical Board of our institution. All subjects were informed and gave their signed consent before their inclusion in the study and samples were anonymized.

Lymphocytes before and after the intake of β-cryptoxanthin-enriched juice were isolated by standard procedures (Lymphoprep™, ATOM, AXIS-SHIELD PoC AS, Norway) and stored in liquid nitrogen. mRNA levels were detected before and after the intervention study using a relative quantification approach in which the amount of
TAP73 and ΔNP73 are expressed in relation to the reference housekeeping gene, succinate dehydrogenase complex subunit A (SDHA). The relative concentrations of TAP73, ΔNP73 and SDHA were calculated by interpolation using a standard curve of each gene plotted from a serial dilution of a cDNA prepared from the RNA of an individual expressing the specific analyzed gene. The differences in expression level of the TAP73 and ΔNP73 in a subject was calculated as the ratio, TAP73 or ΔNP73 after the intervention study:TAP73 or ΔNP73 before the intervention study. A negative sign was added when a decreased in expression was observed.

**β-cryptoxanthin extraction and U-HPLC analysis**

HCT116 cells were seeded on 6-well plates, allowed to attach overnight and treated as described above. Medium culture was collected and cells trypsinized, washed twice in PBS and resuspended in 1 mL of PBS. Twenty microliters of cell suspension was used to count the total cell number (ADAM Cell counter, Digital Bio). Intracellular and extracellular β-cryptoxanthin were extracted and injected onto a U-HPLC as described elsewhere (6).

**RNA extraction and reverse transcription**

Total RNA was extracted using TRIsure reagent (Bioline). RNA amount was measured with a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). For the synthesis of the first-strand cDNA, 400ng of RNA was reverse-transcribed, using the Gold RNA PCR Core Kit (Applied Biosystems). Random hexamers were used as primers for cDNA synthesis.

**PCR array assay**

Expression profiles of genes involved in DNA damage and genes related to p53-mediated signaling pathway were studied with RT² Profiler DNA damage signaling
pathway PCR array (PAHS-029, SABiosciences, CA) and RT² Profiler p53 signaling pathway PCR array (PAHS-027, SABiosciences, CA), respectively. Each kit analyzes the levels of 84 genes sequences. Data were analyzed with software supplied by SABiosciences.

**Real time quantitative PCR**

Quantitative real-time polymerase chain reaction was performed in a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany) using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics). Each reaction was performed in a final volume of 20 µL containing 2 µL of the cDNA product sample, 0.5 µM of each primer and 1x reaction mix including FastStar DNA polymerase, reaction buffer, deoxyribonucleotide triphosphates (dNTPs) and SYBR green.

Primer sets for **TAP73**, **ΔEX2P73**, **ΔEXON2/3P73** and **ΔNP73** and the reaction conditions were as described previously (36). The housekeeping gene succinate dehydrogenase complex subunit A (**SDHA**) was used to normalize gene expression results. The primers used in the mice experiments were: **Sdha**, Forward: 5´ ACCCGGAATTTCGAGACGG 3´ and Reverse: 5´ AGGCACAATCTGATCCTGGC 3´; **TAp73**, Forward: 5´ CTTCAATGAAGGACAGTCTGC 3´ and Reverse: 5´ AATTCTGTTCCCACCTGTGGG 3´; **ΔNp73**, Forward: 5´ TCGGTGACCCCATGAGACAC 3´ and Reverse: 5´ GCGTGCTCCGGGTVGTAGG 3´.

**Cell proliferation assays**

HCT116 cells were treated with vehicle or β-cryptoxanthin 10 µM for 1, 3, 6 and 8 days and then proliferation was evaluated by three approaches: 1) MTT cell proliferation assay (Cayman Chemical Company); 2) cell density was assessed with a
cell-counter apparatus (Digital Bio); and 3) BrdU incorporation was measured by flow cytometry using a FITC-conjugated mouse monoclonal anti-BrdU antibody (BD Pharmigen). Proliferation was calculated from the equation \( N_t = N_0 2^{tf} \), where \( N_t \) is the number of cells at time \( t \), \( N_0 \) is the initial number of cells, \( f \) is the frequency of cell cycles per unit time and \( 1/f \) is the doubling time.

**Apoptosis Assays**

Cells were seeded on a 24-well plate and grown overnight. Cells were pre-treated with vehicle or \( \beta \)-cryptoxanthin for 12 or 24 hours or 6 days. After pre-treatment, cells were treated either with vehicle, \( \beta \)-cryptoxanthin, oxaliplatin at different concentrations or \( \beta \)-cryptoxanthin in combination with oxaliplatin for 36 hours. Culture medium was changed every 24 hours. Floating cells were collected and adherent cells were trypsinized and checked for apoptosis by flow cytometry using the annexin V-FITC/PI (BD Pharmigen) assay.

**Viability Assays**

Cells were seeded on a 96-well plate and grown overnight. Cells were pre-treated and treated as described for apoptosis assays. Cell viability after treatments was tested using the MTT cell viability assay (Cayman Chemical Company).

The synergism between oxaliplatin and \( \beta \)-cryptoxanthin was analyzed by the combination index (CI) obtained using the CalcuSyn software (Biosoft) based on the Chou-Talalay method (37). Four and 5 different concentrations of single oxaliplatin and \( \beta \)-cryptoxanthin, respectively, to test viability for the determination of CI values were evaluated. Viability was also assessed at 4 different combined regimens. CI values < 0.1 indicate very strong synergism, between 0.1-0.3 strong synergism, 0.3-0.7 synergism,
0.7-0.85 moderate synergism, 0.85-0.9 slight synergism, 0.9-1.10 nearly additive and CI values > 1.10 indicate antagonism.

**In vivo treatment**

All experiments were performed in accordance with regulations of the research welfare board of our institution. $10^6$ HCT116 cells and $10^6$ and $10^7$ SW480 cells suspended in 20% matrigel were subcutaneously injected to nude mice (Hsd:Athymic Nude-Foxn1nu, female, 6 weeks old, Harlan Laboratories). SW1417 cell line was not tested since it is supposed to be not tumorigenic in nude mice (ATCC specifications, http://www.lgcstandards-atcc.org/). Mice were randomly assigned to receive one of the following treatments: (i) vehicle (n = 5); (ii) β-cryptoxanthin 10μM (n=7); (iii) vehicle + oxaliplatin 3mg/Kg (n=7) or (iv) a combination of treatments β-cryptoxanthin 10 μM + oxaliplatin 3mg/Kg (n=7). Size of the developing tumor was measured by using vernier calipers. Tumor volume was calculated using the formula $V = 0.5ab^2$, where ‘a’ and ‘b’ indicate the major and minor diameter, respectively. When the volume $V$ in tumor mice measured 75 to 150 mm$^3$, mice were started on daily oral treatment with vehicle (THF 0.2% V/V) or β-cryptoxanthin 10 μM. When the volume $V$ in tumor mice reached 250 to 300 mm$^3$, mice of groups (iii) and (iv) were treated with daily intraperitoneal administration of oxaliplatin (3 mg/Kg) for 5 days, followed by 5 days of rest, for two weekly cycles. After treatment, mice were anesthetized via isofluorane inhalation and peripheral blood was obtained by intracardiac puncture. Finally, mice were sacrificed and their tumors, colon, liver and kidney excised, measured and fixed in 4% paraformaldehyde or stored at -80ºC.
Peripheral blood mononuclear cells were isolated by standard procedures (Lymphoprep™, ATOM, AXIS-SHIELD PoC AS, Norway) and stored in liquid nitrogen.

**Immunohistochemistry analysis**

Immunohistochemistry analysis was performe by standard procedure. See Supplementary “Materials and Methods”.

**Toxicity study**

Body weight was monitored and clinic biochemical parameters as alaninetransaminase (ALT), aspartatetransaminase (AST), gamma-glutaminitransferase, creatinin, bilirrubin and albumin were measured.

**Alkaline Single-cell Gel Electrophoresis (Comet Assay)**

We followed the protocol of Singh et al. (38) (39). See Supplementary “Materials and Methods”.

**Statistical analysis**

The non-parametric Mann-Whitney U test was used to compare independent groups in our *in vitro* and *in vivo* experiments, whilst the Wilcoxon signed ranked test was used for paired data in the intervention study analysis. Two-tailed *p* values ≤ 0.05 were considered statistically significant. Statistical analysis was performed using the SPSS package version 14.0 (SPSS Inc, Chicago, IL).

**RESULTS**

*Cellular uptake of β-cryptoxanthin in HCT116 cells*
We used U-HPLC to measure the intracellular levels of β-cryptoxanthin in HCT116 cells before and after treatment. The net intracellular levels of β-cryptoxanthin after 24 and 48 hours of treatment were 35.444 ± 0.256 and 118.903 ± 0.362 ng/10^6 cells, respectively.

β-cryptoxanthin regulates the levels of the TP53 family members

In order to analyze whether β-cryptoxanthin regulates the expression of genes involved in tumorigenesis, we checked the changes in two RT^2-Profiler™ PCR Arrays (Quiagen) before and after the exposure to β-cryptoxanthin. We performed a p53 signaling pathway PCR array, which includes genes involved in processes of apoptosis, cell cycle, cell growth, proliferation, differentiation and DNA repair. In addition, as β-cryptoxanthin has antioxidant properties, a DNA Damage Signaling Pathway PCR array containing genes implicated in DNA damage response was also performed. Using a threshold value of 2-fold expression change, we found that β-cryptoxanthin affected the expression of a subset of genes in HCT116 cells, with the top 5-deregulated genes in each PCR array presented in Table S1 and Table S2. Both PCR arrays showed up-regulation of TP53 and TP73. We focused our attention on the less known TP73 gene.

β-cryptoxanthin differentially regulates the expression of TP73 isoforms in colon cancer cell lines

The treatment of HCT116 cells with β-cryptoxanthin induced an increase in TAP73 mRNA levels of 2.2- and 4.6-fold at 24 and 48 hours after treatment. By contrast, ∆NP73 mRNA levels decreased 3.57- and 2.77-fold after treatment compared to the control at 24 and 48 hours after treatment. ∆EXON2/3P73 mRNA levels decreased 1.78- and 2.41-fold 24 and 48 after treatment, respectively. Curiously,
ΔEXON2P73 mRNA levels increased 2.71- and 3.55-fold 24 and 48 hours post-treatment. (Fig. 1A)

SW480-ADH cells showed upregulation of TAP73 mRNA levels of 1.8- and 16.9-fold 24 and 48 hours post-β-cryptoxanthin treatment, respectively. In contrast, a decrease of 1.81-fold in ΔNP73 expression after 48 hours was observed. ΔEXON2/3P73 mRNA levels decreased 1.21-fold after 48 hours of treatment and ΔEXON2P73 mRNA levels decreased 1.49-fold 24 hours post-treatment. (Fig. 1B)

In the SW1417 cell line, no significant differences were observed in TAP73 mRNA levels after treatment. However, N-truncated terminal isoforms of TP73 were down-regulated at different times after treatment. Indeed, ΔNP73 mRNA levels decreased 5.9-fold after 48 hours, ΔEXON2/3P73 mRNA levels were reduced 4.0-fold 24 hours post-treatment and ΔEXON2P73 mRNA levels decreased 1.4- and 1.6-fold 24 and 48 hours after treatment (Fig. 1C).

**The expression of TP73 isoforms is regulated after a β-cryptoxanthin intervention study in humans**

Serum levels of β-cryptoxanthin were measured by U-HPLC before and after the intervention study with a β-cryptoxanthin-enriched juice in 20 healthy volunteers. The net levels ranged from 11.6 to 75 μg/dL.

Using a random threshold value of 1.5-fold expression change, we found that the intake of the β-cryptoxanthin-enriched juice affected the expression of TP73 isoforms in 65% of subjects (Fig. 2). Specifically, 20% of subjects presented higher levels of TAP73 in lymphocytes after β-cryptoxanthin intake, 5% presented lower levels of ΔNP73, and 25% showed both effects together. Only in 5% of the subjects did both variants decrease; 5% showed lower levels of TAP73 and 5% presented an increase in...
\(\Delta NP73\) mRNA levels. The downregulation of \(\Delta NP73\) was statistically significant after the \(\beta\)-cryptoxanthin-enriched juice intake \((p=0.04)\).

\textbf{\(\beta\)-cryptoxanthin decreases proliferation in colorectal cancer cells}

HCT116 cells were treated with vehicle or 10 μM \(\beta\)-cryptoxanthin for 1, 3, 6 and 8 days. As shown in Figure 3A, MTT assay revealed that \(\beta\)-cryptoxanthin did not modify cell viability after 1 or 3 days of treatment, but significantly decreased the percentage of living cells 6 and 8 days post-treatment (IC30). Similar results were obtained by measuring cell density with a cell-counter (Fig. 3B). Accordingly, incorporation of BrdU was significantly reduced in HCT116 cells treated with \(\beta\)-cryptoxanthin for 8 days compared to vehicle-treated cells (Fig. 3C). The doubling time calculated for HCT116 cells treated with vehicle was 1.34 days, while the doubling time for HCT116 treated with \(\beta\)-cryptoxanthin was 1.47 days \((p=0.002)\) (Fig. 3D). Levels of \(\Delta NP73\) decreased at these exposure times although no significant differences were observed at 8 days (Fig. 3E), but no differences in \(TAP73\) levels were observed (data not shown). However, 10 μM \(\beta\)-cryptoxanthin administered alone for 6 and 8 days of treatment did not induce apoptosis (Fig. 3F).

To assess whether \(\Delta NP73\) down-regulation by \(\beta\)-cryptoxanthin is involved in its anti-proliferative role, HCT116 cells were stably transfected with a pEGFP-1 plasmid encoding \(\Delta NP73\beta\) or an empty vector, and treated for 6 and 8 days with vehicle or \(\beta\)-cryptoxanthin. After treatment, cell viability was tested by MTT assay. At day 6, cell viability in HCT116 mock cells treated with \(\beta\)-cryptoxanthin was 26\% lower than that in HCT116 mock cells treated with vehicle \((p=0.002)\). However, in HCT116 cells overexpressing \(\Delta NP73\beta\) and treated with \(\beta\)-cryptoxanthin, viability was reduced by only 18\% compared to vehicle treated cells \((p=0.01)\) (Fig. S1A). The difference between
cells overexpressing \( \Delta NP73\beta \) and controls is highly significant \((p=0.003)\). At day 8, viability of HCT116 mock cells treated with \( \beta \)-cryptoxanthin was reduced by 15% compared to those cells treated with the vehicle alone \((p=0.02)\). Remarkably, HCT116 overexpressing \( \Delta NP73\beta \) and treated with \( \beta \)-cryptoxanthin had the same viability as cells treated with the vehicle (Fig. S1B).

\textit{\( \beta \)-cryptoxanthin cooperates with oxaliplatin to induce apoptosis and reduce viability of colon cancer cells}

The oxaliplatin concentration-response experiments for each cell line used in the current study are represented in Fig. S2. HCT116 cells were pretreated for 12 hours with vehicle or 10 \( \mu \)M \( \beta \)-cryptoxanthin, and 100 \( \mu \)M oxaliplatin was then added for 36 hours. Oxaliplatin alone resulted in an apoptosis induction of 61.02% compared with vehicle-treated cells. The combined treatment of oxaliplatin and \( \beta \)-cryptoxanthin induced an 11.5% increase in apoptosis compared with that produced by oxaliplatin alone \((p=0.05)\) (Fig. 4A). Regarding viability, oxaliplatin at 100 \( \mu \)M decreased HCT116 cell viability to 42.4%. The combined treatment reduced cell survival to 37.8% \((p=0.02)\). (Fig. 4B).

Similar results were observed with SW480-ADH and SW1417 cells. Oxaliplatin at 50 \( \mu \)M induced 30.3% apoptosis in SW480-ADH cells. The combination of \( \beta \)-cryptoxanthin and oxaliplatin significantly enhanced apoptosis by 24.6% \((p=0.03)\) (Fig. 4A). Viability assay in these cells showed that oxaliplatin reduced cell survival to 75.63%. The reduction in cell viability induced by the combined treatment was 15% greater than that induced by oxaliplatin alone. \((p=0.03)\). (Fig. 4B).

In SW1417 cells, 100 \( \mu \)M oxaliplatin produced 27.8% apoptosis compared to control cells. When these cells were treated with the combined treatment the percentage...
of apoptosis was 41.6%, which is 13.8% higher than that produced by the treatment with oxaliplatin alone ($p=0.05$) (Fig. 4A). Similar results were obtained with the MTT assay. Oxaliplatin reduced SW1417 cell viability to 88.5%. The combined treatment enhanced oxaliplatin activity by 21.1% ($p=0.03$) (Fig. 4B).

Additionally, the combined treatment was also studied at longer times of exposure to β-cryptoxanthin. HCT116 cells were pretreated with vehicle or β-cryptoxanthin 10 μM for 6 days, and oxaliplatin 50 μM was then added for 2 days. The apoptosis assay revealed that this combined treatment with β-cryptoxanthin and oxaliplatin significantly enhanced the percentage of apoptotic cells by 11.1% ($p=0.03$) compared with oxaliplatin alone (Fig. 4C). In parallel, cell viability was tested by the MTT assay and the combined treatment significantly enhanced the effect of oxaliplatin by 35.1% ($p=0.02$) (Fig. 4C).

According to the Chou-Talalay method (37), the combination of both agents resulted in a synergistic effect exhibiting CI values for HCT116 cells between 0.4 and 0.7 in every combination assay (Fig. S3). Values between 0.3-0.7 were also obtained for SW480-ADH and SW1417 cells (data not shown).

Furthermore, colon cancer cells were treated with different concentrations of oxaliplatin. The combined treatment of oxaliplatin and β-cryptoxanthin led to a similar reduction in cell viability to that achieved by a double dose of oxaliplatin in HCT116 and SW480-ADH cells (Fig. S4).

**Impact of TP53 in response to the combined regimen**

In order to evaluate the impact of TP53 in response to oxaliplatin and the combined treatment, the HCT116 TP53 null cells were used. Similar to the isogenic HCT116 TP53-wild type, β-cryptoxanthin induced TAp73 mRNA levels in 5-folds at 24
hours, and decreased ΔNp73 mRNA expression in 3-folds at 48 hours. As expected, HCT116 TP53 null cells were more resistant to 100 µM oxaliplatin than the wild-type cells, being the viability in the TP53 null cells of 52.3% and 42% in the wild-type (p=0.04) (Fig. 4B). However, no differences were observed in their response to the combined treatment, showing both cell lines a statistical significant reduction of viability compared with the oxaliplatin treatment. The percentage of viability reduction was similar in both cell lines and no statistically different (Fig. 4B and Fig S4A).

**β-cryptoxanthin cooperates with oxaliplatin to induce apoptosis and reduce viability of colon cancer cells through ΔNP73β down-regulation**

HCT116 and SW480-ADH cells were transiently transfected with a pcDNA3.1 plasmid encoding ΔNP73 or the empty vector. Subsequently, we checked the induction of apoptosis after oxaliplatin treatment or combined exposure to this chemotherapeutic agent and β-cryptoxanthin. The combined treatment enhanced the oxaliplatin effect by 11.3% (p=0.04) in HCT116 mock cells and by 8% (p=0.03) in SW480-ADH mock cells, while no differences were seen in either cell line ectopically overexpressing ΔNP73 (Fig. 5A).

Equally, we observed that β-cryptoxanthin combined with oxaliplatin reduced HCT116 and SW480-ADH mock-cell viability by 9.43% (p=0.03) and 15.35 % (p=0.02), respectively, compared with treatment with oxaliplatin alone. No differences in cell survival between the individual and combined treatments were observed in either cell type overexpressing ΔNP73 (Fig. 5B).

**β-cryptoxanthin inhibits tumor growth and cooperates with the anticancer activity of oxaliplatin in vivo**
Animals inoculated with HCT116 cells developed tumors within 8-9 days at approximately 90-95% frequency. No tumors were developed from SW480 cells after one month, although an incipient no measurable mass was observed in the second week after inoculation that either showed involution and disappear or did not grow. Thus, we evaluated the effect of 10 µM β-cryptoxanthin alone or in combination with 3 mg/Kg oxaliplatin on tumor growth in vivo using the HCT116 cells. Nude mice were implanted with HCT116 tumor xenografts and divided into 4 treatment groups: (i) vehicle; (ii) β-cryptoxanthin; (iii) vehicle plus oxaliplatin and (iv) β-cryptoxanthin plus oxaliplatin.

Mice showed a significant decrease in tumor volume after β-cryptoxanthin administration compared with vehicle control group throughout the treatment ($p<0.05$). Oxaliplatin treatment also induced marked tumor suppression ($p<0.05$). The combination of oxaliplatin and β-cryptoxanthin was associated with a significant decrease in tumor volume after the first ($p=0.01$) and the second cycle ($p=0.02$) of treatment compared with animals treated with either agent alone (Fig. 6A). Accordingly, tumors from mice treated with the combined regimen showed a significantly lower number of cells that were positive for the proliferative marker Ki67 than tumors from mice treated with oxaliplatin or β-cryptoxanthin alone ($p=0.02$ and $p=0.04$, respectively) (Fig. 6B). ∆Np73 mRNA levels decreased significantly in the xenograft of mice treated with β-cryptoxanthin administrated alone ($p=0.008$) or in combination with oxaliplatin ($p=0.055$) compared with the control group (Fig. 6C). No differences were observed in TAp73, ΔExon2p73 or ΔExon2/3p73 mRNA levels (data not shown). We evaluated TAp73 and ∆Np73 levels in kidney and liver tissues of mice treated with vehicle and with β-cryptoxanthin and in the colonic mucosa of mice in the 4 groups of treatment. A significant decrease in ∆Np73 expression was observed in the
kidney of mice at the end of the treatment ($p=0.03$). No differences were observed in $TAp73$ levels in kidney either in $TAp73$ and $\Delta Np73$ in the liver (Fig. S5). Finally, $\Delta Np73$ levels were detected in 4 out of 10 mice in the group treated with vehicle, in 4 out of 10 mice treated with β-cryptoxanthin, in 7 out of 10 mice treated with oxaliplatin, and in 6 out of 10 mice treated with the combined regimen. We have observed that $\Delta Np73$ expression was lower in the normal colonic mucosa of mice exposed to β-cryptoxanthin compared with those treated with the vehicle ($p = 0.06$). Similarly, mice treated with the combined regimen showed lower levels of $\Delta Np73$ versus those treated with oxaliplatin in monotherapy ($p = 0.08$) (Fig. S6). No differences in $TAp73$ levels were detected (data not shown).

No differences in body weight or clinical sign of toxicity were observed between vehicle treated control animals and drug treated animals (Fig. S7). In addition, to assess whether β-cryptoxanthin has any genotoxic effect on normal cells, we performed the Comet Assay in mouse peripheral blood mononuclear cells (PBMCs). There were no significant differences in basal DNA damage between control and β-cryptoxanthin groups. As oxaliplatin can induce DNA adduct formation in white blood cells (33), a modified version of the Comet Assay for the measurement of interstrand crosslinks was performed. We found lower formation of DNA adducts in the PBMCs of mice treated with the combination of β-cryptoxanthin and oxaliplatin compared to mice treated with oxaliplatin alone (TM= 4.98±0.14 vs 4.33±1.65; $p=0.04$; TI=28.71±1.78 vs 25.09±1.65; $p=0.055$, respectively) (Fig. S8).

**DISCUSSION**
In the present study we observed that β-cryptoxanthin down-regulates the expression of the oncogenic ΔNP73, which could be associated with the cancer protective effect previously described for this carotenoid. In parallel, we observed inhibition of cell growth by β-cryptoxanthin in vitro and in our animal model. This finding was associated with a decrease in the number of cells in the S-phase. Since no apoptosis was detected in our experiments we conclude that the growth inhibitory effect of this compound is largely due to anti-proliferative activity. Similar data have been reported in lung and stomach cells, suggesting broad anti-proliferative properties for β-cryptoxanthin in both normal and tumor cells (29,30). Interestingly, the overexpression of ΔNP73 abrogates the effect of β-cryptoxanthin, which supports the hypothesis that the negative regulation of this specific variant could be one of the mechanisms underlying the effects of this carotenoid. Nishino and coworkers previously suggested that β-cryptoxanthin may modulate the expression of TP73 (1).

β-cryptoxanthin synergistically enhanced the antitumor effect of oxaliplatin, both in vitro and in vivo. This increase in sensitivity to oxaliplatin was accompanied by the down-regulation of ΔNP73 in colon tumor cells and xenografts. This observation is independent of the TP53 status. The overexpression of ΔNP73 again abolishes this effect, which highlights the role of this TP73 variant in chemoresistance. Thus, we hypothesize that the combined treatment of oxaliplatin and β-cryptoxanthin may be more effective than the oxaliplatin regimen alone in colon cancer patients. The fact that β-cryptoxanthin in monotherapy and in the combined regimen can consistently decreased ΔNP73 levels in kidney and normal colonic mucosa of mice supports its effectiveness in vivo and its plausible activity in different tissues. Moreover, the total dose in our in vivo model was 30 mg/Kg oxaliplatin being similar to the approximately
24-26 mg/Kg administered to CCR patients (administration of 85 mg/m² per 12 cycles, average body surface area 1.8 m², subject of 70-75 Kg), what support its putative translation to the clinical setting. As ΔNP73 up-regulation has been described in most human tumour types, a role for this combined regimen cannot be ruled out in other neoplasias. Furthermore, we report here that the antitumor concentrations of oxaliplatin needed to achieve same percentage of growth inhibition decreased significantly in the presence of β-cryptoxanthin. Therefore, β-cryptoxanthin may reduce the concentration-limiting side effects of oxaliplatin. Other natural-derived compounds in combination with conventional chemotherapeutic agents have also shown efficacy to diminish the toxic concentration of the latter (40). In the same sense, the Comet Assay also revealed that the genomic damage in healthy cells from mice receiving the combined regimen was lower than in those treated with oxaliplatin alone. Significantly, other organs in mice were not affected by the combined treatment. To date, oxaliplatin has a major impact on the management and outcome of colorectal cancer patients but it can induce significant adverse effects, being neuropathy one of the main side effects limiting treatment continuation (33). The administration of β-cryptoxanthin may reduce the effective dose of oxaliplatin, thus moderating the side effects that commonly lead to the interruption of treatment. Moreover, the antitumor activity of β-cryptoxanthin per se is also considerable. β-cryptoxanthin could show a protective effect partially by decreasing oxaliplatin adducts formation. This reduction in DNA damage could lead to a less potent cell death response in the tumor. However, in our in vitro and in vivo approaches we observed that the combined regimen has a stronger anti-tumor activity. Probably, the reduction of ΔNP73 levels in cancer cells can favour the signalling of the damage that is impaired when ΔNP73 is overexpressed (41) and/or modify other not yet described mechanisms through which ΔNp73 can participate in chemo-resistance. It has
to be further evaluated whether the combined treatment proposed here undoubtedly diminish systemic genotoxicity and other oxaliplatin side-effects and the meaning of the \( \Delta NP73 \) downregulation in lymphocytes and other tissues, apart from the tumor, that we have observed in our mice model and in the human intervention study.

Much effort has been devoted last years to the development of targeted therapies in cancer. It seems clear that we will need the combination of several drugs in order to inhibit the multiple signalling networks altered in cancer while keeping toxicities within acceptable limits. Several plant-derived molecules or their analogs have been approved in the last decades for clinical use such as placlitaxel, camptothecin, etoposide and vincristin (42). Currently, more than 30 compounds of natural origin are in different phases of clinical study for the treatment of different types of cancer (43), and many natural compounds are being probed in vitro and in animal models alone and in combination with conventional therapies and their molecular targets identified (40,44–50). Thus, similar approaches to that presented here could be the precursors for pre-clinical trials.

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FIGURE LEGENDS

Figure 1. β-cryptoxanthin differentially regulates the expression of TP73 isoforms in colon cancer cells. Relative TAP73, ΔNP73, ΔEXON2/3P73 and ΔEXON2P73 mRNA levels after 24 and 48 hours of exposure to 10 µM β-cryptoxanthin in (A) HCT116 cells, (B) SW480-ADH cells, and (C) SW1417 cells. * p<0.05, **p<0.01 and ***p<0.001. Mean ± standard deviation of at least three independent experiments is represented.

Figure 2. Modulation of TP73 isoforms expression after a β-cryptoxanthin intervention study in humans. mRNA levels were detected by quantitative real-time PCR in the lymphocytes of each individual before and after the intake. The housekeeping gene succinate dehydrogenase complex subunit A (SDHA) was used for normalization purposes. (A) The graphic represents the differences in TAP73 and ΔNP73 mRNA expression respect to pre-treatment levels which were calculated as the ratio TAP73 or ΔNP73 after intervention:TAP73 or ΔNP73 before intervention. (B) Quantification representation of the data. The negative sign symbolizes decrease expression post-intervention.

Figure 3. β-cryptoxanthin at a concentration of 10 µM decreases proliferation in HCT116 cells after 6 and 8 days of treatment. (A) Cell proliferation measured by MTT assay, (B) cell density measured with a cell-counter apparatus, (C) quantification of BrDU incorporation (left) and representative plots (right), (D) doubling time calculation, (E) quantitative real-time PCR analysis of ΔNP73 mRNA levels after treatment and, (F) apoptosis assay by flow cytometry analysis using Annexin V-FITC/IP staining. *p<0.05, **p<0.01. Mean ± standard deviation of at least three independent experiments is represented.
Figure 4. β-cryptoxanthin at a concentration of 10 µM cooperates with oxaliplatin at 100 µM for HCT116 TP53 wild-type, HCT116 TP53 null and SW1417 cells and 50 µM for SW480 to induce apoptosis and reduce viability. (A) Apoptosis assays by flow cytometry analysis using AnnexinV-FITC/IP staining after 72 hours of treatment. Quantification of four independent experiments *p<0.05, **p<0.01 (left) and representative plots (right). (B) Quantification of cell viability by MTT assay after 72 hours of treatment . *p<0.05, **p<0.01. (C) Quantification of apoptosis in HCT116 cells treated with vehicle, 10 µM β-cryptoxanthin, 50 µM oxaliplatin or the combined regimen by flow cytometry analysis and representative plots (left) and quantification of cell viability (right) after 8 days of treatment. Mean ± standard deviation of at least three independent experiments is represented.

Figure 5. (A) Apoptosis assay by flow cytometry (Annexin V-FITC/IP) and, (B) MTT cell viability assay in HCT116 and SW480-ADH cells ectopically overexpressing ΔNP73. * p<0.05, **p<0.01. 10 µM β-cryptoxanthin and 50 µM oxaliplatin were used for the individual and combined treatments. Mean ± standard deviation of at least three independent experiments is represented.

Figure 6. 10 µM β-cryptoxanthin inhibits tumor growth and cooperates with 3 mg/Kg oxaliplatin in vivo. When the volume in tumor mice reached 75 to 150 and 250-300 mm³ β-cryptoxanthin and oxaliplatin, respectively, were administered. (A) Analysis of xenograft tumor volume at the start point, at the end of the first cycle of treatment, at the end of the 5 days of rest and at the end of the second cycle of treatment. (B) Ki67 immunohistochemistry in xenograft tumors after the two cycles of treatment, representative images (upper panel) and quantification (lower panel). (C) Relative
mRNA levels of ΔNp73 in xenograft tumors after the two cycles of treatment. *p=0.05, **p<0.01. Mean ± standard deviation of 7 mice per group is represented.

REFERENCES


- Figure 1 -
**Figure 2**

- A graph showing the relative mRNA levels for TAp73 and ΔNp73 across 37 subjects.

- A table summarizing the fold-change in TAp73 and ΔNp73 mRNA levels for each subject.
Figure 5
β-CRYPTOXANTHIN SYNERGISTICALLY ENHANCES THE ANTITUMORAL ACTIVITY OF OXALIPLATIN THROUGH ΔNP73 NEGATIVE REGULATION IN COLON CANCER

Coral San Millan, Beatriz Soldevilla, Paloma Martín, et al.

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