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Effect of carotenoids on nutrient uptake by MDA-MB 231 cells

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Resumo:

O cancro da mama é a maior causa de mortalidade relacionada com o cancro em mulheres mundialmente. O cancro da mama do subtipo triplo negativo (ou basal), caracterizado pela ausência de recetores de estrogénio, recetores de progesterona e HER2, apresenta um comportamento agressivo e uma resposta baixa à terapia molecular atual. O desenvolvimento e a progressão do cancro, que é dependente de altos níveis de energia e macromoléculas, têm vindo a ser associados com a reprogramação de algumas vias metabólicas, incluindo a da glucose (efeito "Warburg"), glutamina ("dependência de glutamina") e ácido fólico (importante para o metabolismo de unidades de 1-carbono). Os carotenoides, com atividade anticancerígena demonstrada, são conhecidos por interferir com a proliferação celular, apoptose, progressão do ciclo celular, angiogénese e metastização. O objetivo deste estudo foi caracterizar o efeito anti-tumoral de diferentes carotenoides numa linha celular humana de cancro da mama triplo negativo (células MDA-MB-231), e investigar se a interferência com a captação celular de nutrientes poderá ser um mecanismo envolvido no seu efeito anti-tumoral. Verificou-se que quatro carotenoides distintos (β-caroteno, crocina, fucoxantina e astaxantina) foram capazes de alterar a proliferação celular, crescimento da cultura, migração e viabilidade celular das células MDA-MB-231. Dos compostos testados, o β-caroteno demonstrou ter o efeito mais interessante, promovendo uma diminuição da proliferação, viabilidade e migração celular e do crescimento da cultura. O efeito deste composto foi posteriormente caracterizado. Verificou-se que o β-caroteno aumentou significativamente a captação de glucose (^{3}H-DG) e diminuiu a captação de glutamina (^{3}H-GLN) independente de sódio. A captação de ácido fólico (^{3}H-FA), a qual não é dependente do pH, não foi afetada pelo β-caroteno. O β-caroteno não alterou os níveis de stress oxidativo, como verificado pelos níveis de peroxidação lipídica e pela quantificação da carbonilação proteica. Para além disso, o β-caroteno afetou o ciclo celular nas células MDA-MB-231, promovendo o aumento de células na fase S, e a diminuição de células na fase G1. Também se demonstrou que o efeito anti-proliferativo do β-caroteno está associado com a ativação da via de sinalização intracelular JNK, e que este carotenoide é capaz de aumentar o efeito anti-proliferativo da doxorrubicina, tendo portanto um efeito aditivo. Verificou-se ainda que o efeito do β-caroteno é seletivo para as células de cancro, dado que este composto não afeta a viabilidade celular, proliferação e migração das células MCF12-A (uma linha não tumoral de epitélio mamário humano).

Em conclusão, o β-caroteno apresenta um efeito anti-tumoral em relação a células de cancro da mama triplo-negativo, o qual não está correlacionado com uma diminuição da captação celular de nutrientes (glucose, glutamina e ácido fólico) nem com um efeito antioxidante. No entanto, este efeito é seletivo para células tumorais, está dependente da ativação da via de sinalização intracelular JNK e está relacionado com uma alteração no ciclo celular. O β-caroteno apresenta, portanto, potencial como co-adjuvante da doxorrubicina no tratamento do cancro de mama basal, dado que o seu efeito é seletivo para as células de cancro e potencia o efeito anti-proliferativo da doxorrubicina nas células de cancro.

Palavras-chave: MDA-MB-231, MCF12-A, cancro da mama, carotenoides, β-caroteno, doxorrubicina

Abstract:

Breast cancer is a major cause of cancer-related mortality in women worldwide. Triple negative or basal subtype of breast cancer, characterized by the absence of ER (estrogen receptors), PR (progesterone receptors) and HER2, presents an aggressive behavior and a poor response to current molecular targeted therapy. Cancer development/progression, which is dependent on high energy and macromolecules supply, is associated with the reprogramming of some metabolic pathways, including pathways of glucose ("Warburg effect"), glutamine ("glutamine addiction") and folic acid (important for one-carbon metabolism) metabolism. Carotenoids, with anti-cancer activity, are known to interfere with proliferation, cell cycle progression, apoptosis, angiogenesis and metastization. The aim of this study was the characterization of the antitumor effect of carotenoids on a triple-negative human breast cancer cell line (MDA-MB-231 cell line), and investigation of interference with nutrient cellular uptake as a mechanism contributing to their antitumoral effect. We verified that four distinct carotenoids (β-carotene, crocin, fucoxanthin, astaxanthin) interfere with cell proliferation, culture growth, migration and viability of MDA-MB-231 cells. Of the tested compounds, β-carotene presents the most interesting antitumoral effect, as it decreased cell viability, proliferation, migration and culture growth. The effect of this compound was further characterized. We verified that β-carotene significantly increased ^{3}H-deoxy-D-glucose uptake and decreased Na^{+}-independent ^{3}H-GLN (glutamine) uptake. ^{3}H-FA (folic acid) uptake, which was found to be pH-independent, was not affected by β-carotene. β-carotene did not change oxidative stress levels, as assessed by lipid peroxidation levels and carbonyl protein levels. Moreover, β-carotene affect cell cycle of MDA-MB-231 cell line, by promoting an increase of cells on S phase and a decrease of cells on G1 phase.We further verified that the anti-proliferative effect of β-carotene involves activation of the JNK intracellular pathway, and that it was able to enhance the anti-proliferative effect of doxorubicin. Moreover, β-carotene did not affect cell viability, proliferation and migration rates of MCF-12A cells, a non-tumoral human epithelial cell line, revealing that the effect of β-carotene is cancer cell-selective.

In conclusion, β-carotene presents an anti-tumoral effect in relation to a triple-negative breast cancer cell line, which is not correlated with a decrease in the cellular uptake of nutrients (glucose, glutamine and folic acid) nor with an antioxidant effect, but which is cancer cell-specific, dependent on activation of the JNK intracellular pathway and involves interference with cell cycle. β-carotene thus presents a potential as co-adjuvant to doxorubicin for basal breast cancer treatment, because it has a cancer cell-specific effect and potentiates the anti-proliferative effect of doxorubicin in cancer cells.

Keywords: MDA-MB-231, MCF12-A, breast cancer, carotenoids, β-carotene, doxorubicin

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Abbreviations

^{3}H-GLN - ^{3}H-Glutamine

^{3}H-DG - ^{3}H-Deoxy-d-glucose

^{3}H-FA - ^{3}H-Folic acid

ASCT2 - Alanine, serine, cysteine transporter 2

ATF4 - transcription factor 4

ATP - Adenosine triphosphate

BRAC1 - Breast cancer gene 1

BRAC2 - Breast cancer gene 2

BSA - Bovine serum albumin

BMI - Body mass index

cDNA - Complementary DNA

CoA - Coenzyme A

DCA - Dichloroacetate

DHFR - Dihydrofolate reductase

DMSO - Dimethyl sulfoxide

DNA - Desoxyribonucleic acid

DOX - Doxorubicin

EDTA - Ethylenediaminetetraacetic acid

ER - Estrogen receptor

FBS - Fetal bovine serum

GLUTs – Facilitative glucose transporters

GLUT1 - Facilitative glucose transporter 1

HER2 - Human epidermal growth factor receptor 2

LAT1 - Large neutral aminoacid transporter 1

LDH - Lactate dehydrogenase

mTOR (mammalian target of rapamycin)

MTHFR - Methylenetetrahydrofolate reductase

MTHFD2 - (Methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 2)

NADH - Nicotinamide adenine dinucleotide

PI - Propridium iodide

PR - Progesterone receptor

RFC – Reduced folate carrier

ROS - Reactive oxygen species

SAM - S-adenosylmethionine

SGLTs - Sodium glucose cotransporters

SRB - Sulforhodamine B

TCA cycle – Tricarboxylic acid cycle

THF - Tetrahydrofolate

TNBC - Triple negative breast cancer

Figure Index

INTRODUCTION

Breast cancer

Breast cancer is a disease where breast cells mutate, originating an uncontrolled grow and division. Breast cancer development (Figure 1) is associated with the following steps: increased proliferation (hyperplasia); proliferation of atypical cells (atypical hyperplasia); *in situ* carcinoma (non-invasive cancer, associated with a better prognosis and a major survival) and invasive carcinoma [1].

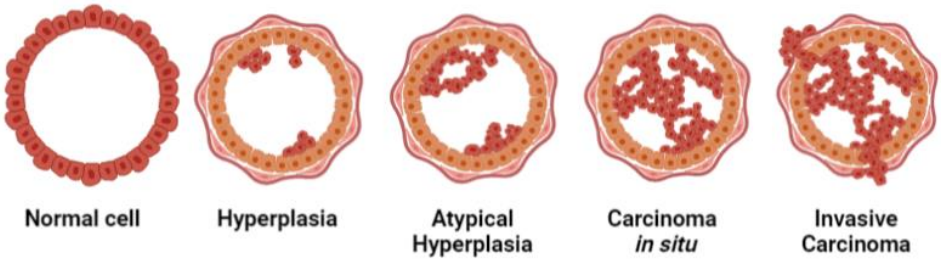

Figure 1- Breast cancer evolution, from hyperplasia (increased proliferation), atypical hyperplasia (benign histological lesions with risk for breast cancer development), carcinoma in situ (a carcinoma without invasiveness capacity) to an invasive carcinoma.

Epidemiology

Breast cancer is one of the most common malignancies, becoming in 2020 the most incident cancer worldwide. For both sexes, breast cancer is the fifth more lethal cancer worldwide, after lung, colorectal, liver and stomach cancer (Figure 2). Relatively to females, it is the major cause of cancer-related mortality worldwide [2, 3]. In Europe, according to GLOBOCAN 2020, it is the third major cause of cancer-related mortality, after lung and colorectal cancer, in both sexes (Figure 2) [3].

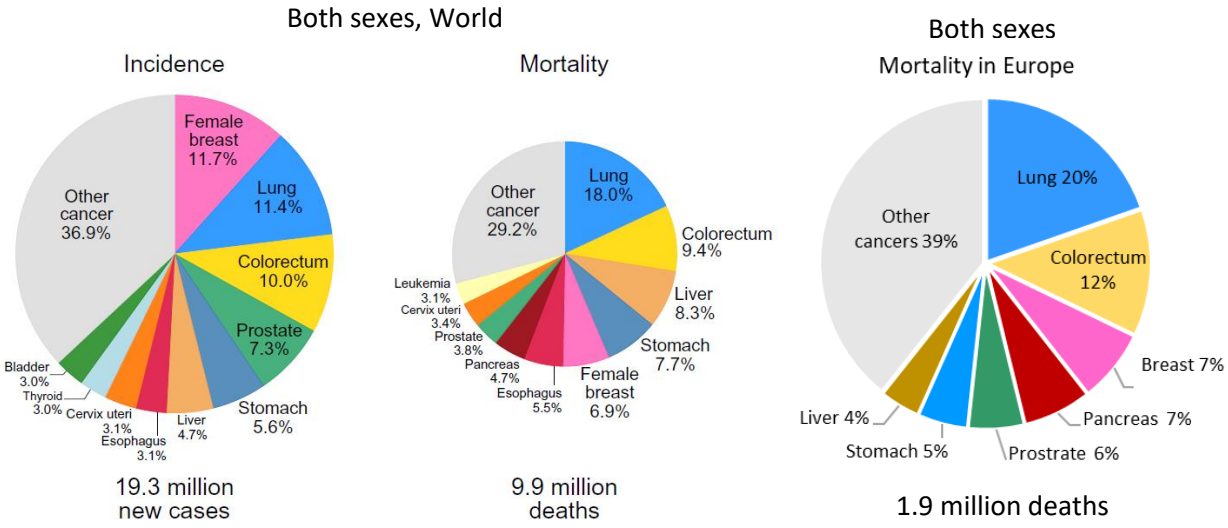

Figure 2- Distribution of cases (in world) and deaths (in world and in Europe) for the 10 most common cancers in 2020 for both sexes. Taken from [3]

Breast cancer classification

Breast cancer is a heterogeneous disease, a differentiation based in distinct gene expression profiles, markers of proliferation and biomarkers [4].

Breast cancers can be distinguished into four major intrinsic subtypes: luminal A, luminal B, HER2 (human epidermal growth factor receptor 2)-positive and basal type (or triple negative - TNBC) [2]. These subtypes are categorized according to proliferation (Ki-67) and immunohistochemistry biomarkers [5].

Luminal A, the most common subtype, has an increased expression of progesterone receptors (PR) and low expression of Ki-67, is estrogen receptor (ER)-positive and HER2-negative. Luminal B is PR/ER-positive and has high expression of Ki-67. The presence or absence of HER2 subdivides Luminal B subtypes [4]. HER 2 is HER-positive and ER and PR-negative [4]. Finally, the TNBC subtype is characterized by the absence of ER and PR and no overexpression of HER2 protein [2].

Breast cancer risk factors

Development of breast cancer is associated with DNA damage and the main associated risk factors are age (risk increases from age of 30 until 80 years), gender (female), personal and family history of breast cancer and genetic risk factors (presence of BRCA1 and BRCA2 gene mutations) [6, 7]. Diet also plays an important role on increasing the risk, through the decreased consumption of fruits, vegetables and antioxidants (carotenoids and vitamin C) and the increased consumption of processed or red meat [8]. Moreover, a balanced diet contributes to maintenance of a normal body mass index (BMI), and a high BMI is associated with an increased risk of breast cancer initiation in post-menopausal women [8]. Long exposure to sexual hormones (related to consumption of oral contraceptives), nulliparity or low parity and early menarche also contribute to an increased risk for the development of breast cancer [8].

Triple negative breast cancer is responsible for 15–20% of diagnosed breast tumours, with a higher incidence in young and African American women, and is often associated with BRCA germline mutations [2].

Breast cancer treatment

Tumour staging, grading and intrinsic subtype are factors that influence the choice of treatment of human breast cancer [4]. Endocrine therapy benefits more the Luminal cancers with high expression of ER and for aggressive Luminal B (without HER2 overexpression), chemotherapy is recommended [4]. Trastuzumab, a targeted anti-HER2 therapy, in conjugation with chemotherapy is used on non-luminal tumours with high expression of HER2 [4]. The absence of ER on TNBC results in the insensitivity of this breast cancer subtype to anti-hormonal treatment; indeed, this cancer subtype still presents limited treatments, because of the absence of targeted therapies [4, 9]. The first-line treatment applied in TNBC, actually, is chemotherapy [4]. The most frequent regimens for chemotherapy contain taxanes or anthracyclines (including doxorubicin) or the combination of both [4].

Hallmarks of cancer

The hallmarks of cancer (Figure 3), which consist on cellular characteristics that enable cancer cell development and cancer progression, comprise a sustained proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [10]. For cancer development, high energy and nutrient levels are required, including glucose, glutamine and folic acid [11]. To acomplish these needs, cancer cells exhibit a reprogramming of some metabolic pathways, also designed deregulation of cellular energetics. The cellular metabolic reprogramming was recently included in the hallmarks of cancer cells [12] (Figure 3).

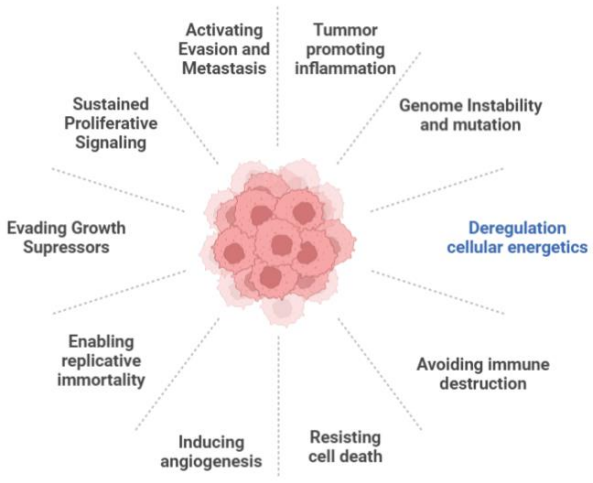

Figure 3 -Hallmarks of cancer. These consist on cellular characteristics that enable cancer cell development and cancer progression.

Nutrients and breast cancer

Glutamine

Glutamine is a nutrient essential for proliferation and progression of cancer, and is also a source of nitrogen, which is essential to synthesize non-essential amino acids, and contributes to redox balance, through glutathione synthesis [13]. In more detail, glutathione neutralizes peroxide free radicals; these, when in excess, cause cellular damage [14]. Glutamine also is essential to produce ATP through the TCA (tricarboxylic acid) cycle [14]. For this to occur, glutamate, which is obtained from glutamine deamidation, is converted into α-ketoglutarate that is then incorporated in the TCA cycle [14]. The mTOR pathway can be deregulated in various types of cancer and glutamine can also regulate this pathway, as a source of amino acids that are able to stimule mTOR activity [14].

The cellular transporters for glutamine on breast cancer cells are ASCT2 (SLC1A5), $ATB^{0,+}$ (SLC6A14), and LAT1(SLC7A5) [15]. ASCT2 (alanine, serine, cysteine transporter 2) is a Na^+-coupled transporter and a target of the oncogene c-Myc, which by activating this glutamine transporter, promotes glutamine uptake [13, 16]. ASCT2 is overexpressed in some cancers and associates with poor prognosis, being used as a prognostic marker [17]. Another glutamine transporter is LAT1, a Na^+-independent cotransporter that is associated with CD98 [15]. Triple negative breast cancer cells have an increased need of glutamine - a “glutamine addiction” - that could be a potential therapeutic target [13]. In comparison, luminal-type cells have significantly lower ASCT2-mediated glutamine uptake [13]. Presently, the therapeutic strategies based on “glutamine addiction” focus on glutaminase inhibition, but LAT1 and ASCT2 can be a potential target for chemotherapeutic drugs [14, 15].

Glucose

Glucose can be sinthetized by human organism or obtained trought food ingestion, and is the main energetic subtrate used by cancer cells [11, 18]. When in presence of anaerobic conditions, glucose is oxidized through glycolysis, originating pyruvate and NADH [10]. Pyruvate can then be converted into lactate in the citoplasm [10], or included in the TCA cycle, under aerobic conditions [10]. Cancer cells exhibit a metabolic reprogramming that includes a metabolic shift from mitochondrial oxidative phosphorylation to aerobic glycolysis (Warburg effect), although both pathways can create ATP [12]. This effect is characterized by high lactate production and increased glycolytic rates [12].

Glucose has specific transporters in cells, including cancer cells: sodium glucose cotransporters (SGLTs) and facilitative glucose transporters (GLUTs) [18, 19]. GLUTs are subdivided in 3 classes: I (1,2,3 and 4 – the classic transporters), II (GLUT5 and GLUT7 – fructose transporters) and III (GLUT6, 8, 10, 12 and 13) [18].

GLUT 1 transporter is higly expressed in various types of tumours, inclunding breast, hepatic, ovarian and coloretal cancer [20]. GLUT1 is also highly expressed in MDA-MB-231 and MCF-7 breast cancer cell lines [21, 22].GLUT1 levels has a prognostic and diagnostic marker and its expression is increased on the presence of RAS and Src oncogenes [20, 23]. Expression of GLUT1 is associated with an aggressive behavior, high proliferation and energy requirements, and consequently poor prognosis and reduced survival rates [20, 23].

Folic Acid

One-carbon metabolism (Figure 4) is essential for cancer cells, as it is involved in DNA synthesis and methylation process, in holding DNA integrity, gene expression and cellular homeostasis and supporting the growth and proliferation of the cells, through the production of purine nucleotides, ATP, NADH and glutathione [24, 25].

On that process, the non-essential amino acids glycine and serine are the source of one-carbon units (methyl and methylene groups, for example) that can enter the methionine cycle and the folate cycle [25, 26].

In the folate cycle, folic acid is reduced to dihydrofolate, and consequently, it is converted to the active form tetrahydrofolate (THF) by the action of DHFR (dihydrofolate reductase) [25, 26]. THF generates methyl-THF, when carbons units from serine are added to the reduced form, through the action of SHMT2 (serine hydroxymethyltransferase 2) [25, 26]. Methyl-THF can participate in redox transformations and in the synthesis of nucleobases [25, 26]. The folate cycle also contributes to the supply of high energy, through *de novo* generation of ATP (by promoting the synthesis of adenosine) and promotes the regeneration of NADH and NADPH [27].

In the methionine cycle, the co-substrate for SAM-dependent methyl-transferases, SAM (S-adenosylmethionine), is produced [27]. Serine metabolism is also connected with the synthesis of SAM [27]. When the folate-derived product 5,10-methylene-tetrahydrofolate (5,10-methylene-THF) is converted into 5-methyl-THF, through the action of methylenetetrahydrofolate reductase (MTHFR), the re-methylation of homocysteine occurs [27]. In this reaction, homocysteine, a methionine-derived compound, which is also a precursor for glutathione and cysteine synthesis, is re-methylated, resulting in methionine synthesis, in a reaction also using the cofactor vitamin B_{12} (Figure 4) [25-27].

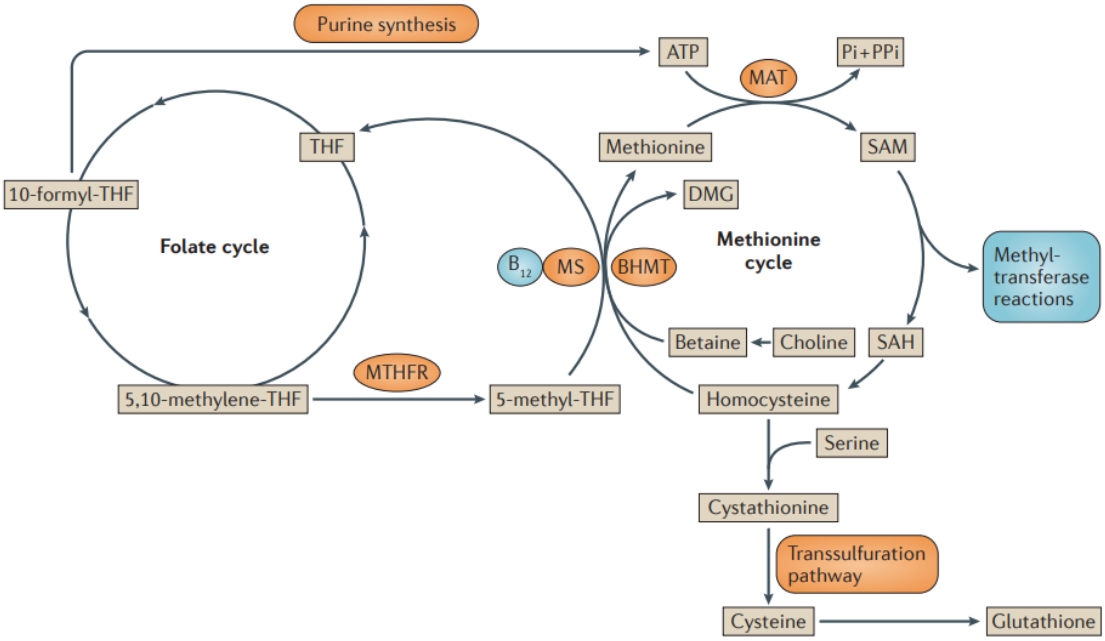

Figure 4- One-carbon metabolism: the Methionine and the Folate Cycles. In the Methionine cycle, the co-substrate SAM ((S-adenosylmethionine) is produced from methionine by S-adenosylmethionine synthase (presented in the Figure as MAT(methionine adenosyltransferase)). In the Folate cycle, folic acid is reduced to dihydrofolate, and consequently converted to the active form tetrahydrofolate (THF) by the action of DHFR (dihydrofolate reductase). When the folate-derived product 5,10-methylene-tetrahydrofolate (5,10-methylene-THF) is converted into 5-methyl-THF, through the action of methylenetetrahydrofolate reductase (MTHFR), the re-methylation of homocysteine occurs. DMG, dimethylglycine; B_{12}, vitamin B_{12}; MS, methionine synthase; BHMT, betaine-homocysteine S-methyltransferase; SAH, S-adenosylhomocysteine Taken from [27]

mTOR (mammalian target of rapamycin) is involved in the regulation of one carbon metabolism, through the activation of the transcription factor 4 (ATF4) [25]. ATF4 enhances the mitochondrial pathway of folate cycle, through the regulation of expression of MTHFD2 (Methylenetetrahydrofolate dehydrogenase (a $NADP^+$-dependent enzyme) 2) [25]. mTOR pathway, in the presence of high nutrient amounts, enhances anabolic reactions, supporting cell proliferation and growth [25].

An increase in flux of one carbon-metabolism is observed when the oncogene MYC hijacks regulatory pathways. Moreover, constitutive KRAS activation, promoted by oncogenic mutations, is associated with an increased expression of folate cycle enzymes. Also, the tumor suppressor gene p53 can interfere with one carbon metabolism, through the regulation of serine synthesis [25]. Moreover, this flux is regulated by the action of folate carriers, folate receptors and ATP-binding cassette transporters. For cellular folate uptake, the reduced folate carrier (RFC1/SLC19A1) was the first transporter described [28], but other folate transporters, such as the folate transporter proton-coupled folate transporter (PCFT/SLC46A1), were subsequently described [28]. Cellular folate uptake also involves folate receptors, the third component that regulates the cellular uptake of folate, which is composed by three receptors with high affinity to folate (FRα, FRβ and FRγ) [28].

In accordance with the importance of folate for cancer cells, targeting folate-mediated one-carbon metabolism is a strategy used in cancer therapy, through antifolates [25, 28]. Antifolates affect folic acid absorption and metabolism, with a consequent cellular death. For example, the anticancer agent methotrexate (MTX) targets dihydrofolate reductase (DHFR), resulting in increased dihydrofolate levels on the cell that leads to inhibition of nucleotide biosynthesis and to cell death, through the ability to target and inhibit folate-dependent enzymes [28, 29]. Other antifolates are described in the literature and clinically available, such as raltitrexed, lometrexol and pemetrexed [28].

Carotenoids

Carotenoids, organic pigments synthesized by plants, macroalgae, insects and fungi, are tetraterpenoids with antioxidant properties, anticancer activity (chemopreventive and chemotherapeutic capacity) and protective effects in relation to cardiovascular diseases [30]. Carotenoids are subdivided in carotenes (lycopene, carotene) and xanthophylls (lutein, zeaxanthin, neoxanthin and astaxanthin) [30]. Lycopene, lutein, zeaxanthin, astaxanthin, β-carotene, crocin and fucoxanthin are carotenoids included in the human diet [30].

Carotenoids have a significant impact in decreasing the oncogenic progress through modulation of oxidative stress, cell cycle progression, apoptosis, angiogenesis, and metastasis, by the action of oncogenic proteins (including Ras, Bcl-2 and cyclins) and anti-oncogenic proteins (including, p27, p21 and p53) [30]. For instance, β-carotene was found to induce apoptosis, increase caspase 3-activity, and decrease the expression of anti-apoptotic (Bcl-2 and PARP) and survival (NF-kB) proteins in MCF-7 breast cancer cells [31]. Also on MCF-7 cells, cytochrome *c* release and mitochondrial dysfunction were observed due to β-carotene-induced production of ROS (reactive oxygen species) [32].

Carotenoids can act on multiple cancer cell intracellular signalling pathways, including the PI3K/AKT/mTOR pathway, p38, ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinases). For instance, astaxanthin, β- and α-carotene and lycopene inhibit these pathways. Also, Janus kinase (JAK)-signal transducer, the activator of transcription proteins (STAT) and the Wnt/ β-catenin can be blocked by carotenoids. Moreover, carotenoids such as lycopene inhibit Wnt/β-catenin and Jak1/STAT3 pathways (Figure 5) [30].

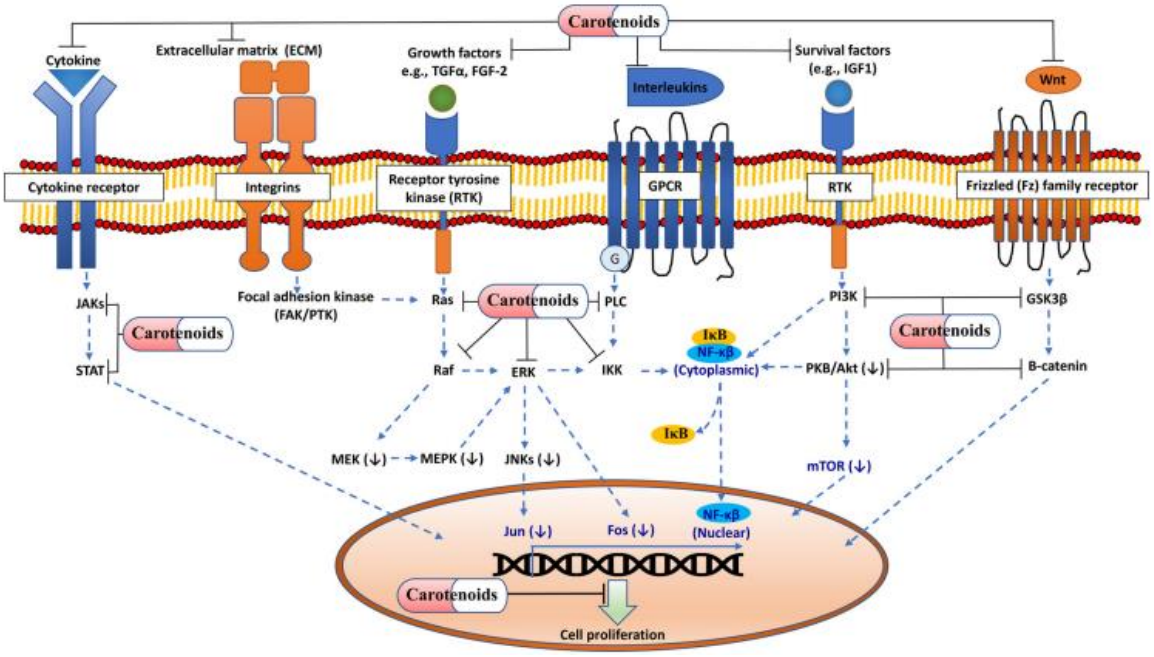

Figure 5- The impact of carotenoids on key regulatory points in oncogenic pathways. ↑, upregulation; ↓, downregulation; ⊥, suppression. JAK/STAT, Janus kinase-signal transducer and the activator of transcription proteins; TGFα, transforming growth factor alpha; FGF-2, basic fibroblast growth factor; IGF1- insulin-like growth factor 1; GPCR, G protein-coupled receptor; PI3K, Phosphoinositide 3-kinase; mTOR, the mammalian target of rapamycin. Taken from [30]

AIMS

The aim of this study is to evaluate and characterize the potential antitumor effect of carotenoids on triple-negative breast cancer cells and to investigate if interference with nutrient cellular uptake can contribute to their antitumoral effect. For this, we will first evaluate the effect of four different carotenoids (β-carotene, crocin, fucoxanthin and astaxanthin) on cell proliferation, culture growth, migration and viability of a triple negative cell line (MDA-MB-231 cells). After this evaluation, we will select the carotenoid with the most interesting effect to test its effect on glucose (^{3}H-DG), glutamine (^{3}H-GLN) and folic acid (^{3}H-FA) uptake, in order to investigate if interference with nutrient uptake can also contribute to its antitumoral effect. We will also conduct a more complete characterization of its potential antitumor effect (by evaluating its effect upon cell morphology, cell cycle, oxidative stress levels, and also by investigating the signaling pathways involved in its antitumor effects). Moreover, its effect in combination with doxorubicin and its effects in a non-tumoral breast epithelial cell line (MFC-12A) will be also evaluated.

MATERIALS AND METHODS

Materials

^{3}H-Deoxy-D-glucose (2-[1,2-^{3}H(N)]-deoxy-D-glucose: specific activity 60 Ci/mmol), ^{3}H-L-glutamine, (L-[2,3,4-^{3}H]; specific activity 60 Ci/mmol), ^{3}H-folic acid [3,5,7,9-3H] (specific activity 40 Ci/mmol) (American Radiolabeled Chemicals Inc., St Louis, MO, USA). ^{3}H-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany). Heat-inactivated horse serum (Gibco, Life Technologies Corporation, CA, USA). D-glucose, hydrochloric acid (HCl), 2-propanol, triton X-100, trichloroacetic acid (TCA) (Merck, Darmstadt, Germany). Acetic acid (Scientific Laboratory Supplies, Wilford, United Kingdom). DMEM/F12 medium (1:1) (catalogue P04-41150; PAN-Biotech, Aidenbach, Germany). Antibiotic/antimycotic solution (100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B), bovine serum albumin (BSA), fetal bovine serum (FBS), astaxanthin, β-carotene, crocin, fucoxanthin, reduced nicotinamide adenine dinucleotide (NADH), RPMI 1640 medium, sodium hydroxide (NaOH), sodium pyruvate, sulforhodamine B (SRB), tris-(tris(hydroxymethyl)-aminomethane hydrochloride), trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Sigma, St. Louis, MO, USA). Doxorubicin hydrochloride, LY294002, SP600125, PD98059, SB203580, Dinitrophenylhydrazine (DNPH), guanidine (Sigma), PI (propridium iodide)

Cell culture

The MDA-MB-231 cell line (a triple negative human breast epithelial cancer cell line) and the MCF-12A cell line (a non-tumorigenic human breast epithelial) were obtained from the American Type Culture Collection (ATCC) and used between passage number 53 to 78 (MDA-MB-231) and 33 to 37 (MCF-12A). MDA-MB-231 cells were grown in RPMI 1640 medium (catalogue #R6504, Sigma, St Louis, MI, USA) supplemented with 2 mM L-glutamine, 10 mM sodium bicarbonate, 15% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic/antimycotic solution. MCF-12A cells were maintained in DMEM/F12 medium (1:1) supplemented with 20 ng/ml human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone, 5% heat-inactivated horse serum and 1% antibiotic/antimycotic solution.

Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2. Culture medium was changed every 3-4 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 4 min, 37°C), split 1:3 ratio and sub-cultured in plastic culture dishes (21 cm^{2}; diameter 60 mm; TPP®, Trasadingen, Switzerland).

For determination of cell viability, proliferation, migration and culture growth, cells were seeded on 24-well plastic cell culture dishes (2 cm^2 Ø 16 mm; TPP®) and used at 90% confluence (4 days-old culture). For ^{3}H-glutamine (^{3}H-GLN), ^{3}H-deoxy-d-glucose (^{3}H-DG) and ^{3}H-folic acid (^{3}H-FA) uptake experiments, cells were seeded similarly and used at 100% confluence (5-days old culture). For protein carbonyl content and TBARS assay, cells were seeded on 12-well plastic cell culture dishes (3.60 cm^2; TPP®) and used at 100% confluence (5-days old culture). For determination of cell cycle cells were seeded in plastic culture dishes (21 cm^2; diameter 60 mm; TPP®, Trasadingen, Switzerland).

Treatment of the cells

The carotenoids used in this study were: astaxanthin, β-carotene, crocin and fucoxanthin. The concentrations of carotenoids (1 µM, 10 µM, 100 µM and 500 µM) and doxorubicin (10 µM) to be tested were chosen based on previous works of our group [33]. MDA-MB-231 and MCF-12A cells were treated for 24h with carotenoids and/or other compounds in serum-free culture medium. Astaxanthin and β-carotene were dissolved in DMSO, crocin and doxorubicin were dissolved in H_2O and fucoxanthin in ethanol. For controls, the solvent of each compound was used. The final concentration of these solvents in culture medium was 1% (v/v).

Incorporation of ^{3}H-thymidine assay – determination of cell proliferation rates

Cellular proliferation rates were determined by quantification of the incorporation of ^{3}H-thymidine, that quantifies DNA synthesis. MDA-MB-231 and MCF-12A cells were exposed for 24 h to carotenoids or doxorubicin (or their solvents) and incubation with ^{3}H-thymidine 0.025 µCi/mL was done in the last 5 h of treatment. The excess of ^{3}H-thymidine was rinsed off with 10% TCA, followed by a 20 min incubation with NaOH (280 µL/well). Before the addition of scintillation fluid, the lysate was neutralized with 5 mM HCl. Radioactivity was quantified by liquid scintillometry (LKB Wallac Liquid Scintillation Counter 1209 Rackbeta liquid scintillator). Results are expressed as incorporation of ^{3}H-thymidine (µCi/mg protein).

Sulforhodamine B assay - determination of culture growth

Sulforhodamine B has the capacity to electrostatically bind to basic amino acids of cell culture proteins previously fixated with TCA (trichloroacetic acid). It measures whole-culture protein content as an index of culture growth. After the 24 h treatment with carotenoids (or their solvents), culture medium was replaced with 500 µL PBS 1x and 62.5 µL of ice cold 50% (w/v) TCA was added to each well to fix cells, for 1 h at 4°C in the dark. Then, cells were washed with tap water to remove excess TCA, air dried, and then stained for 15 min with 125 µL 0.4% (w/v) SRB dissolved in 1% acetic acid per well. After SRB removal, cells were rinsed with 1% acetic acid to remove residual die, and then air dried again. The bound die was solubilized with 375 µL of 10 mM Tris-NaOH solution (pH=10.5). To obtain absorbance (540 nm) values below 0.7, the contents of each well were diluted and placed in a 96-well plaque where the absorbance was measured using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

LDH assay - determination of cell viability

Cell death is correlated to leakage of lactate dehydrogenase (LDH) to the extracellular medium and measurement of LDH activity, through the measurement of the decrease in absorbance of NADH during the reduction of pyruvate to lactate, can be used to evaluate cell viability.

MDA-MB-231 cells were submitted to a 24 h treatment with carotenoids and/or other compounds (or their solvents). After this period, the extracellular medium was removed and 50 µL of extracellular medium was added to 250 µL of a mixture of phosphate/pyruvate (50 mM/0.63 mM) solution and NADH (11.3 mM) solution. Absorbance values were measured in a 96-well plaque at 340 nm, and the decrease in absorbance value, in this time interval, was calculated.

LDH activity was expressed as the percentage of extracellular activity in relation to total cellular LDH activity, which represents 100% cell death. To determine the total activity, control cells were solubilized with 500 µL of 0.1% (v/v) Triton X-100 and incubated for 1 h at 4°C, and then the sample was processed in the same way as the others.

Injury assay – determination of cell migration rates

The injury assay was performed in order to evaluate the effect of the carotenoids on the migration rates of MDA-MB-231 cells. Cells were submitted to treatment with carotenoids (or the solvent), during 24 h. At 0 h (beginning of treatment), a scratch in the cell monolayer was made with a sterile 10 µL pipette tip, to cause a lesion. Images were obtained at 0 h and after 24 h of treatment. The quantification of migration area was performed using ImageJ software.

^{3}H-glutamine (^{3}H-GLN), ^{3}H-deoxy-d-glucose (^{3}H-DG) and ^{3}H folic acid (^{3}H-FA) uptake assays

For ^{3}H-glutamine (^{3}H-GLN) and ^{3}H-deoxy-d-glucose (^{3}H-DG) uptake measurements, the experiments were performed in GF-HBS buffer (glucose-free HEPES buffered saline) (composition, in mM: 140 NaCl, 5 KCl, 20 HEPES, 2.5 $MgSO_4$, 1 $CaCl_2$; pH 7.4). Sodium-independent ^{3}H-GLN uptake was determined by using GF-HBS buffer in which NaCl was replaced with 140 mM LiCl. For ^{3}H-folic acid uptake measurement, the experiments were performed in HMBS buffer (HEPES-MES buffered saline) (composition, in mM: 140 NaCl, 5 KCl, 12.5 HEPES, 12.5 MES, 5 glucose, 1.2 $MgSO_4$, 1.2 $CaCl_2$; pH 7.5 or 5.5).

Initially, the culture medium was removed, and the cells were washed with 300 µL GF-HBS or HMBS buffer at 37°C. Then cell monolayers were preincubated for 20 min in GF-HBS or HMBS buffer at 37°C. Uptake was then initiated by the addition of 200 µL buffer at 37°C containing 5 nM ^{3}H-GLN, 10 nM ^{3}H-DG or 20 nM ^{3}H-FA. When tested, carotenoids (or the solvents) were present during both pre-incubation and incubation periods. Incubation was stopped after 6 min, by removing the incubation medium, placing the cells on ice, and rinsing the cells with 500 µL ice-cold GF-HBS or HMBS buffer. The cells were then solubilized with 300 µL 0.1 % (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4), and placed at 4°C overnight. The radioactivity of the samples was quantified by liquid scintillometry (LKB Wallac Liquid Scintillation Counter 1209, Turku, Finland).

Determination of oxidative stress-induced cellular damage

TBARS (thiobarbituric acid-reactive substance) assay

After a 24h treatment of MDA-MB-231 cells with β-carotene (10 µM) (or its solvent), an homogenization solution (KH_2PO_4 62.5 nM, $Na_2HPO_4.2H_2O$ 50 nM, Triton X-100 0.1 %, pH 7) was added to each sample and cells were resuspended. The reaction was started by the addition of 200 µL TCA 50% to each sample, followed by a centrifugation for 2 min at 10000 rpm (4ºC). Then, 1% 2-thiobarbituric acid (TBA) was added to the supernatant and an incubation was carried out for 40 min at 95 ºC. After that, 300 µL of each sample was transferred to a 96-well culture plate, and the colored complex was quantified spectrophotometrically at 535 nm using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The results were normalized to the protein content.

Protein Carbonyl content assay

MDA-MB-231 cells were treated for 24 h with β-carotene (10 µM) (or its solvent). At the end of the treatment, the medium was removed and the cells were washed with PBS solution. The cells were then resuspended in homogenization solution and 100 µL of this solution were added to a microtube, in duplicates, and 200 µL of TCA 10 % were added. The samples were then centrifuged at 13000 rpm for 2 min at 4 °C and the supernatant was discarded. The duplicates were treated with 500 µL of DNPH (dinitrophenylhydrazine) or 500 µL HCl and rested for 1 h. After that, 500 µL of TCA 20 % at 4 °C were added, the samples were kept on ice for 15 min and then centrifuged at 13000 rpm for 2 min at 4 °C. Then, a wash with ethanol:ethyl acetate (1:1) for two times was done, with centrifugations in between at 13000 for 2 min at 4 °C. We next dissolved the sediment overnight with 500 µL of guanidine 6 M at 4 °C, followed by a centrifugation at 3000 rpm for 15 min. The absorbances were then measured with a microplate reader at 340 nM (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Cell Cycle

Changes in cell distribution across cell cycle stages were assessed by measurement of DNA content in the cells. Cells were treated for a 24 h with β-carotene (10 µM) (or DMSO) on 21 cm^2 plates. At the end of the treatment, the medium was removed and the cells were washed with PBS solution. Further, cells were removed enzymatically (0.25% trypsin–EDTA, 4 min, 37°C), resuspended in 3 mL medium without serum, and submitted to a centrifugation (5 min, 300g). The supernatant was removed and the pellet was re-suspended in 200 µL of 70% ethanol, and then stored at -20ºC. Next, cells were washed in PBS with 2% BSA and re-suspended in 100 µL of propidium iodide (PI) solution (PI/Rnase, Immunostep) for 15 min at room temperature. Flow cytometry analysis was performed on a BD Accuri C6 flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Results were analysed using FlowJo version 10.7.1 software (Ashland, Oregon). Results are expressed as % of total cells arrested in each phase in the cell cycle.

Protein determination

The protein content of the cell monolayers was determined as described as Bradford [34], using human serum albumin as standard.

Statistics

Arithmetic means are given with standard error of the mean (SEM). The value of n indicates the number of replicates of at least two different experiments. Statistical significance of the difference between three or more groups was evaluated by one-way ANOVA test, followed by the Student-Newman-Keuls test. For comparison between two groups, Student t test was used. Differences were considered significant when $P < 0.05$. Analyses were done using the GraphPad Prism version 9.0 software (San Diego, CA, USA).

RESULTS

Effect of carotenoids on cell growth, viability, proliferation and migration of MDA-MB-231 cells

In the first series of experiments, the effects of four different carotenoids (β-carotene, crocin, astaxanthin and fucoxanthin) on culture growth, cell viability, cell proliferation and migration ability of MDA-MB-231 cells were evaluated.

Culture growth was evaluated using the SRB assay. Astaxanthin and fucoxanthin showed no effect on culture growth. In contrast, β-carotene (all tested concentrations, with no concentration-dependency observed) and crocin (1 and 100 µM) significantly decreased culture growth (Figure 6).

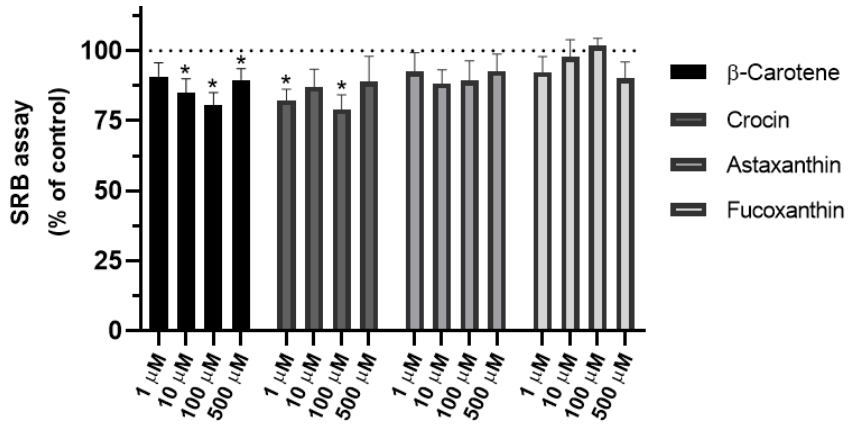

*Figure 6-Effect of exposure to carotenoids on culture growth. After a 24h exposure to carotenoids (1-10-100-500 µM) or solvent, MDA-MB-231 culture growth was determined by cellular protein quantification using the sulforhodamine B (SRB) assay (n=9-12). Results are present presented as absorbance (% of control). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05)*

Cell viability was evaluated using the LDH assay. As shown in Figure 7, fucoxanthin had no statistically significant effect on cell viability. On the other hand, β-carotene (500 µM) decreased cell viability, and crocin (10-100-500 µM) and astaxanthin (500 µM) increased it.

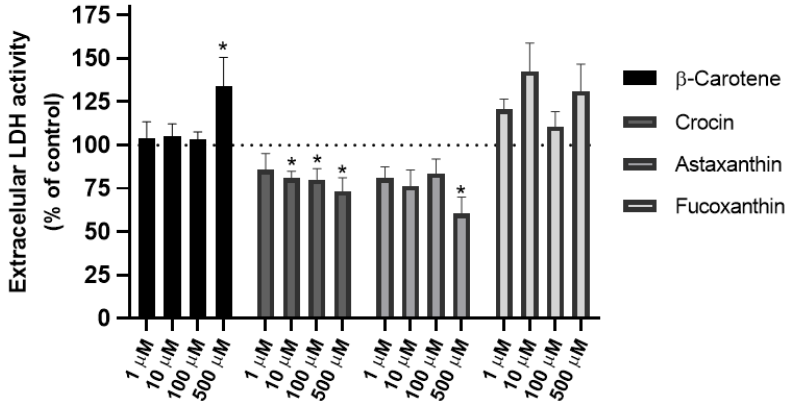

*Figure 7-Effect of exposure to carotenoids on cell viability. After a 24h exposure to carotenoids (1-10-100-500 µM) or solvent, MDA-MB-231 cell viability was determined by quantification of extracellular LDH activity (n=9-12). Results are presented as LDH activity (% of control). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05)*

Cell proliferation was evaluated by quantification of ^{3}H-thymidine uptake. β-carotene decreased cell proliferation (all concentrations tested, with no concentration dependency observed). Moreover, astaxanthin (10-500 µM) and crocin (10 and 500 µM) also decreased cell proliferation. In contrast, fucoxanthin (1-10 µM) caused an increase in cell proliferation (Figure 8).

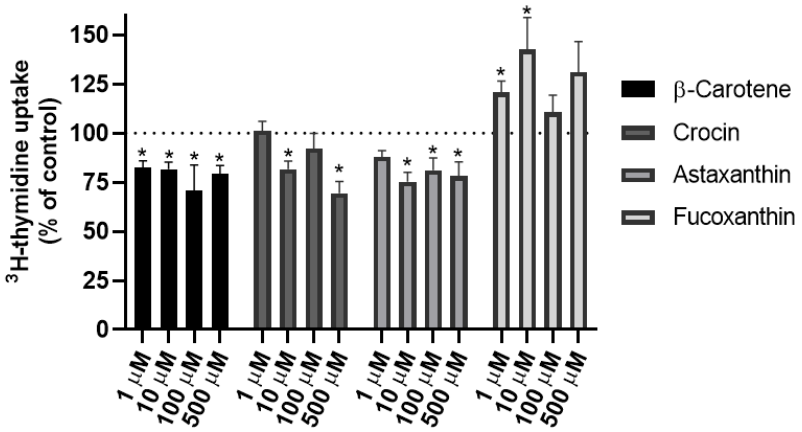

*Figure 8-Effect of exposure to carotenoids on cell proliferation rates. After 24h exposure to carotenoids (1-10-100-500 µM) or solvent, MDA-MB-231 cell proliferation rate was determined by quantification of ^{3}H-thymidine incorporation (n=9-12). Results are presented as µCi/ mg protein (% of control). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05)*

Cell migration was evaluated using the injury assay (Figure 10). Astaxanthin and fucoxanthin showed no statistically significant effects on cell migration. However, β-carotene (500 µM) and crocin (10 and 500 µM) decreased cell migration.

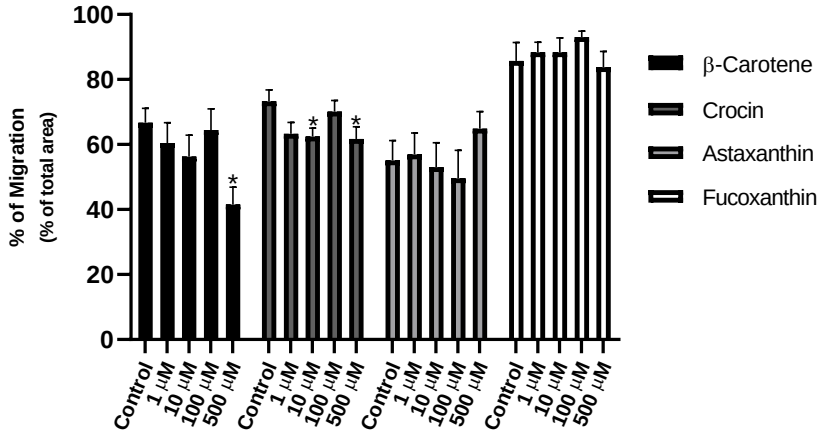

*Figure 9-Effect of exposure to carotenoids on cell migration. A scratch in cell culture was made at 0 h, and cells were then exposed to the carotenoids (1, 10, 100 or 500 µM), or to the solvent, for 24h. Pictures were taken at 0h and 24h (n=6-9). Results are presented as percentage of migration (% of total area). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05)*

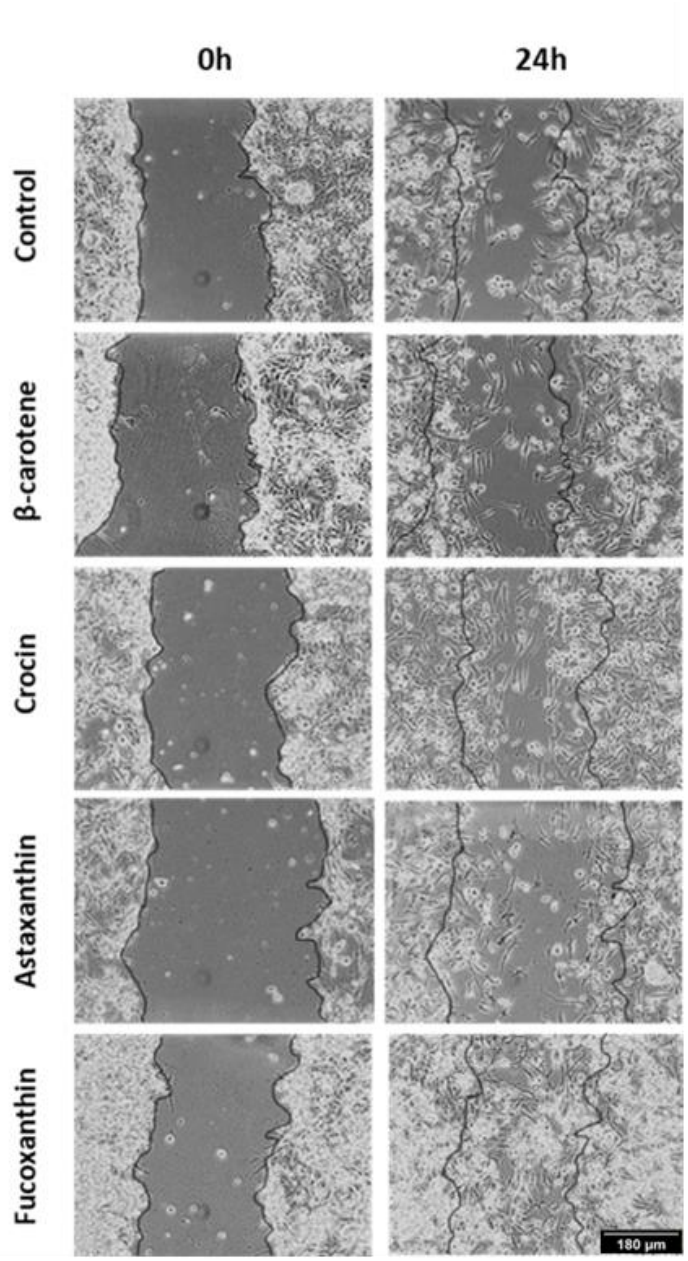

Figure 10 - Representative images of cell migration, evaluated by the injury assay. A scratch in cell culture was made at 0 h, and cells were then exposed to the carotenoids (1, 10, 100 or 500 µM), or to the solvent, for 24h. Pictures were taken at 0h and 24h. Shown are the representative images obtained with each carotenoid (500 µM) (magnification 10 X).

Based on the results from the previous assays, we selected β-carotene as the most interesting carotenoid, because it decreases cell viability and proliferation, culture growth and migration ability of MDA-MB-231 cells. This reveals an antitumoral effect. So, further assays were made to characterize this effect.

Effect of β-carotene on ^{3}H-glutamine (^{3}H-GLN), ^{3}H-deoxy-d-glucose (^{3}H-DG) and ^{3}H folic acid (^{3}H-FA) cellular uptake by MDA-MB-231 cells

^{3}H-GLN uptake

In this series of experiments, the effects of β-carotene on the cellular uptake of ^{3}H-GLN, ^{3}H-DG and ^{3}H-FA by the MDA-MB-231 cell line were evaluated.

The MDA-MB-231 cell line was previously shown to take up ^{3}H-GLN in a time-dependent manner, mainly by a Na^+-dependent mechanism [35], and this was confirmed in this study (Figure 11). In relation to total and Na^+-dependent ^{3}H-GLN uptake, β-carotene showed no significant effect. In contrast, β-carotene (1 µM) slightly decreased Na^+-independent ^{3}H-GLN uptake (Figure 12).

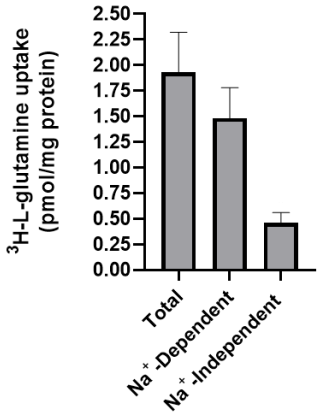

Figure 11- ^{3}H-GLN uptake by MDA-MB-231 cells, determined in the presence (Total) and absence of Na^+ (Na^+-independent). Na^+-dependent uptake was calculated by subtracting Na^+-independent from total uptake. Results are presented as pmol/mg protein (absolute values). Shown are arithmetic means ±SEM (n=4).

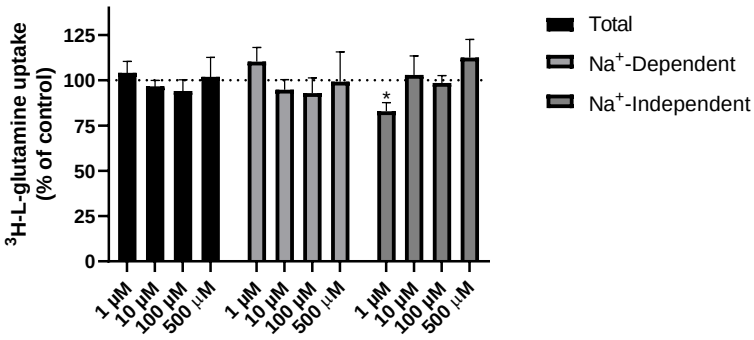

*Figure 12-Effect of β-carotene on ^{3}H-GLN cellular uptake. Cells were exposed to β-carotene (1-10-100-500 µM) or solvent for 24h. Afterwards, cells were preincubated for 20 min followed by an incubation for 6 min with 5 nM ^{3}H-GLN (with or without Na^+), also in the presence of β-carotene or solvent (n=6). Results are presented as pmol/mg protein (% of control). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05).*

^{3}H-DG uptake

The MDA-MB-231 cell line is known to take up ^{3}H-DG in a time-dependent manner [36]. As shown in Figure 12, β-carotene (500 µM) was able to increase ^{3}H-DG uptake.

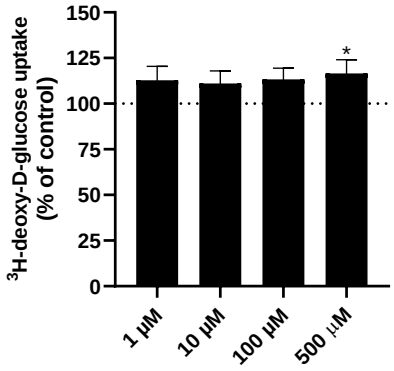

*Figure 13-Effect of β-carotene on ^{3}H-DG cellular uptake. After a 24h exposure to β-carotene (1-10-100-500 µM) or solvent, cells were preincubated for 20 min followed by an incubation for 6 min with 10 nM 3H-DG (n=16). Results are presented as pmol/mg protein (% of control). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05)*

^{3}H-FA uptake

The time-course of ^{3}H-FA (^{3}H-folic acid) accumulation by MDA-MB-231 cells was first determined. For this, cells were incubated with 20 nM ^{3}H-FA for various periods of time at two different pH: 7.5 and 5.5. ^{3}H-FA uptake was found to be time-dependent and pH-independent (Figure 14). A linear ^{3}H-FA uptake was observed in the first 6 min of incubation at both pH. With this information, we selected 6 min incubation time as the standard time and pH=7.5 to perform further experiments.

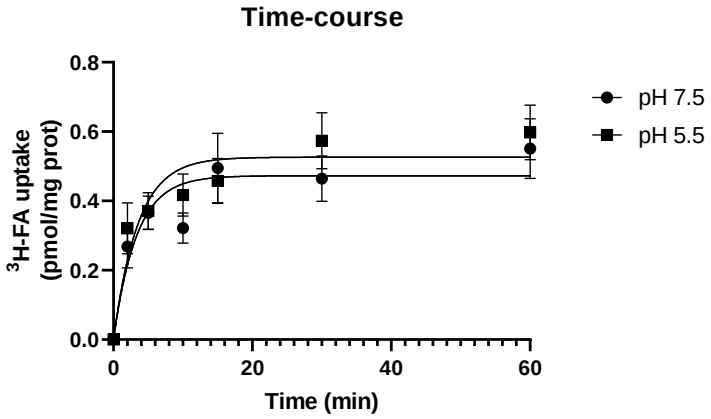

Figure 14-Time-course of ^{3}H-folic acid (^{3}H-FA) uptake by MDA-MB-231 cells. Cells were incubated at 37°C with 20 nM ^{3}H-FA for 2, 5, 10, 15, 20 and 60 min, at two different extracellular pH (pH=5.5/pH=7.5). Shown are arithmetic means ± SEM (n=8)

Next, we evaluated the effect of β-carotene on the cellular uptake of ^{3}H-FA. As seen in Figure 15, β-carotene did not affect ^{3}H-FA uptake.

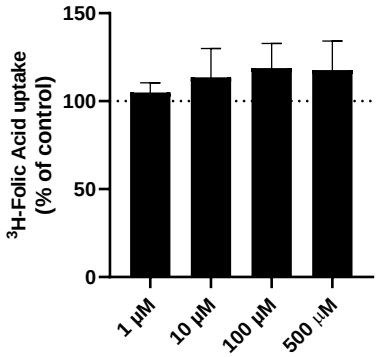

*Figure 15-Effect of β-carotene on ^{3}H-FA cellular uptake. After a 24h exposure to β-carotene (1-10-100-500 µM) or solvent, cells were preincubated for 20 min followed by an incubation for 6 min with 20 nM ^{3}H-FA (n=12), also in the presence of β-carotene or solvent. Results are presented as pmol/mg protein (% of control). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05)*

For further characterization of the antitumor effect of β-carotene, only the 10 µM concentration was tested. So, we next evaluated the effect of β-carotene (10 µM) on oxidative stress levels, cell morphology and cell cycle. Moreover, the putative involvement of some intracellular signalling pathways on its antitumoral effect was evaluated, as well the effect of β-carotene (10 µM) on the MCF-12A non-tumoral breast epithelial cell line, to determine if its effects are cancer-selective. Finally, the effect of β-carotene (10 µM) on the antitumor effect of doxorubicin was also evaluated.

Effect of β-carotene on oxidative stress levels in MDA-MB-231 cells

In order to determine the effect of β-carotene on oxidative stress levels in MDA-MB-231 cells, we quantified lipid peroxidation (TBARS) and protein carbonyl levels. In relation to these assays, we verified that β-carotene (10 µM) had no effect (Figure 16).

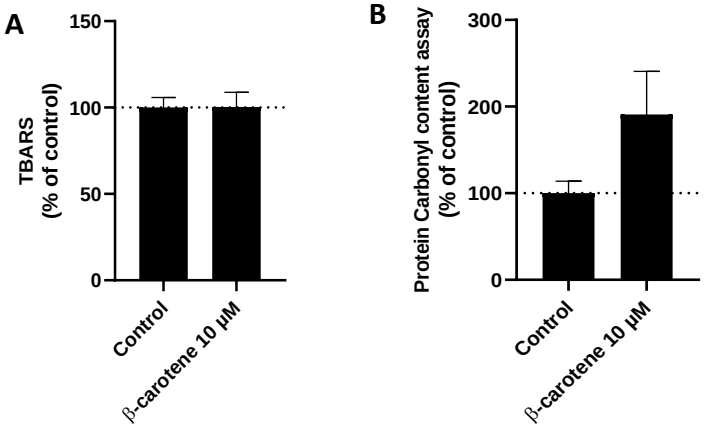

*Figure 16-Effect of β-carotene on oxidative stress levels. (A) After a 24h exposure to β-carotene (10 µM) or solvent, lipid peroxidation levels were determined using the TBARS assay (n=9). (B) After a 24h exposure to β-carotene (10 µM) or solvent, protein carbonyl levels were determined using the Protein Carbonyl content assay (n=12). Shown are arithmetic means ±SEM. * Significantly different from control (DMSO) (P<0.05)*

Effect of β-carotene on MDA-MB-231 cell morphology

We observed that β-carotene (10 µM) slightly modifies the cell morphology of MDA-MB-231 cells (Figure 17). Indeed, an irregular morphology of β-carotene- treated cells (Figure 17B), compared to normal spindle-shaped morphology in 2D cultures (Figure 17A), was observed [37].

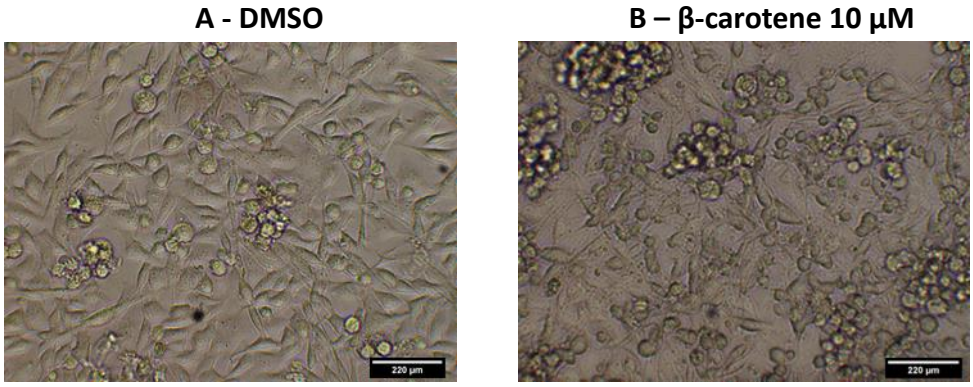

Figure 17- Effect of β-carotene on morphology of MDA-MB-231 cells. After a 24 h exposure to β-carotene (B) (10 µM) or solvent (A), the cell culture was visualized (magnification 20 X).

Effect of β-carotene on MDA-MB-231 cell cycle

We also tested the effect of β-carotene on cell cycle distribution. The cell cycle distribution on solvent (DMSO)-treated MDA-MB-231 cells was 62 % in G1 phase, 26 % in S phase and 12 % in G2/M phase. The cell cycle distribution on β-carotene-treated MDA-MB-231 cells was 50 % in G1 phase, 30 % in S phase and 13 % in G2/M phase (Figure 18A). So, as shown in Figure 18B, cell cycle was affected by β-carotene (10 µM), through an increase in the amount of cells retained in S phase and a decrease of cells in G1 phase. These results indicate that β-carotene induces cell cycle arrest at S phase.

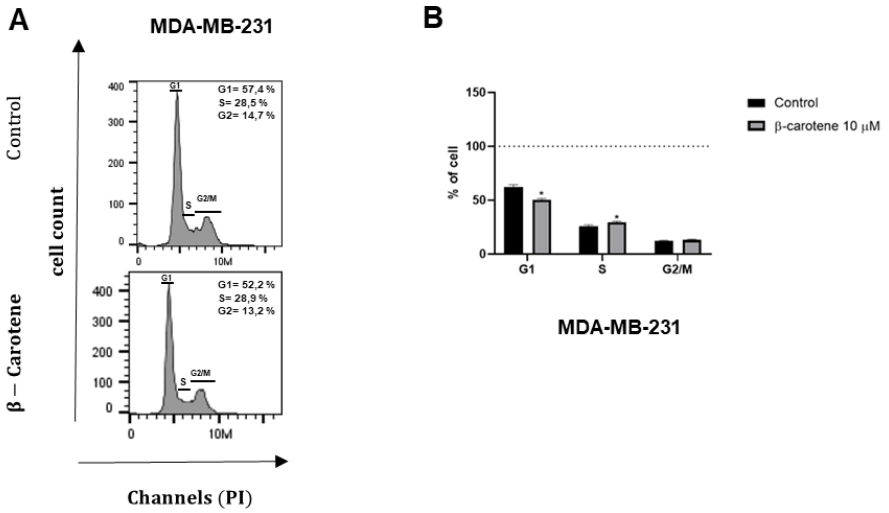

*Figure 18-Effect of β-carotene (10 µM) on MDA-MB-231 cell cycle distribution (n=5-6). Shown are arithmetic means ± SEM.*Significantly different from control (P < 0.05).*

Determination of the putative involvement of the intracellular signalling pathways PI3K, ERK/MEK1/MEK2, p38 MAPK and JNK on the antiproliferative effect of β-carotene in MDA-MB-231 cells

In relation to the mechanism involved in the antitumoral effect of β-carotene, we decided to investigate if the intracellular signalling pathways PI3K, ERK/MEK1/MEK2, p38 MAPK and JNK were involved in the antiproliferative effect of β-carotene. We verified that the antiproliferative effect of β-carotene was abolished in the presence of SP 600125 (5 µM), but was not affected by the other inhibitors, namely LY294002, PD98059 and SB203580 (Figure 19). LY294002, PD98059 and SB203580 are specific inhibitors for PI3K, ERK/MEK1/MEK2 and p38 MAPK intracellular signalling pathways, respectively. These results thus suggest that the antiproliferative effect of β-carotene involves activation of the JNK pathway, but not of the other pathways (PI3K, ERK/MEK1/MEK2 and p38 MAPK).

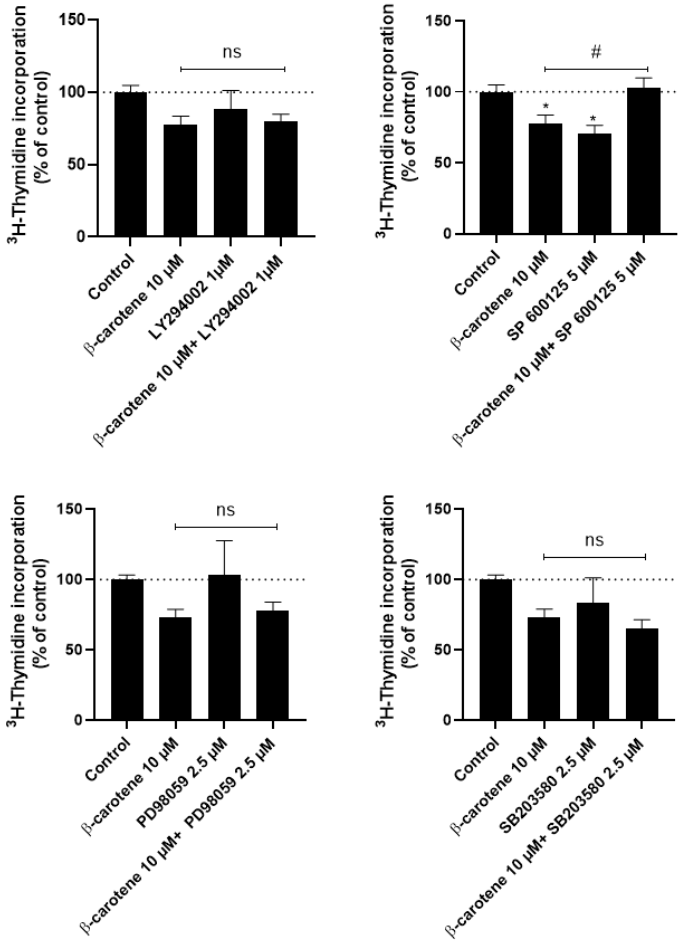

*Figure 19- Effect of inhibitors of the intracellular signalling pathways PI3K (LY294002), ERK/MEK1/MEK2 (PD98059), p38 MAPK (SB203580) and JNK (SP 600125) on the antiproliferative effect of β-carotene. After a 24h exposure to β-carotene (10 µM) (or solvent) and/or inhibitors, cell proliferation rates were determined (n=9). Results are presented as µCi/ mg protein (% of control). Shown are arithmetic means ±SEM. * Significantly different from control (P<0.05), # Significantly different from each other; ns not different*

Effect of β-carotene on MCF-12A cells

In this series of experiments, we investigated the effect of β-carotene (10 µM) on the viability, proliferation, migration rates and cell cycle of the MCF-12A non-tumoral cell line. As shown in Figure 20, cell viability, proliferation, migration rates and cell cycle were not affected by β-carotene (10 µM) in these cells.

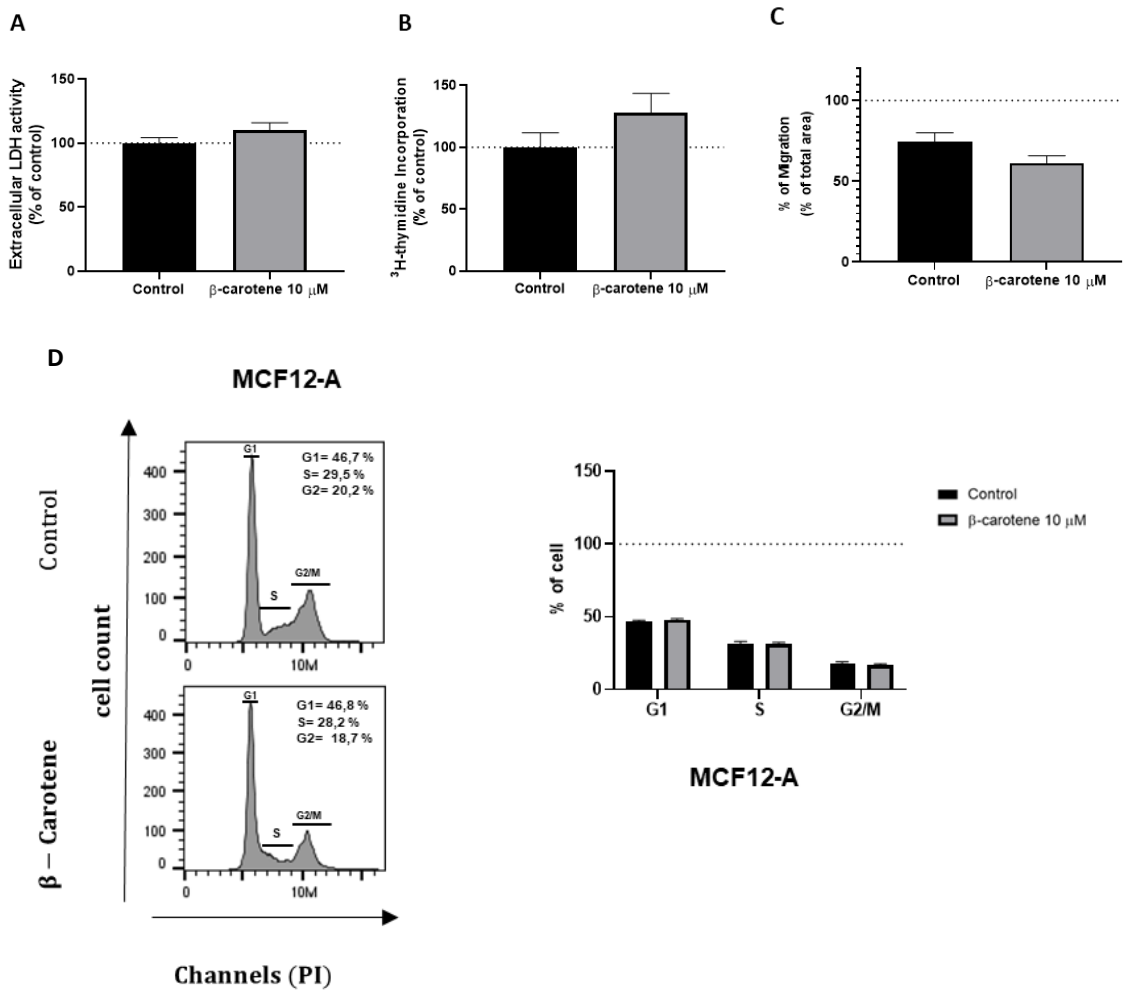

*Figure 20 - Effect of a 24 h exposure to β-carotene on MCF-12A cell viability, proliferation, migration rates and cell cycle. (A) Effect of β- carotene cell viability (n=8-12). Results are presented as LDH activity (% of control). (B) Effect of β-carotene on proliferation rates. Results are presented as ^{3}H-thymidine incorporation (µCi/mg protein) (% of control). (n=9). (C) Effect of β-carotene on cell migration rates. Results are presented as % of total area (n=9). (D) Effect of β-carotene on cell cycle distribution (n=5-6). Shown are arithmetic means ± SEM. *Significantly different from control (P < 0.05).*

Effect of β-carotene in combination with doxorubicin on MDA-MB-231 cell proliferation

In this final series of experiments, we investigated the combined effect of β-carotene (10 µM) and doxorubicin (10 µM), the most commonly chemotherapeutic drug used on breast cancer treatment, on MDA-MB-231 viability, proliferation and migration. In relation to cell viability (Figure 21A) and migration (Figure 21B), we verified that the effect of doxorubicin was not statistically different from the effect of the combination of β-carotene and doxorubicin. This allow us to conclude that β-carotene does not potentiate neither the cytotoxic nor the anti-migratory effect of doxorubicin. In contrast, combination of β-carotene with doxorubicin enhances the anti-proliferative effect of doxorubicin (Figure 21C). These data suggest an additive anti-proliferative effect of β-carotene and doxorubicin.

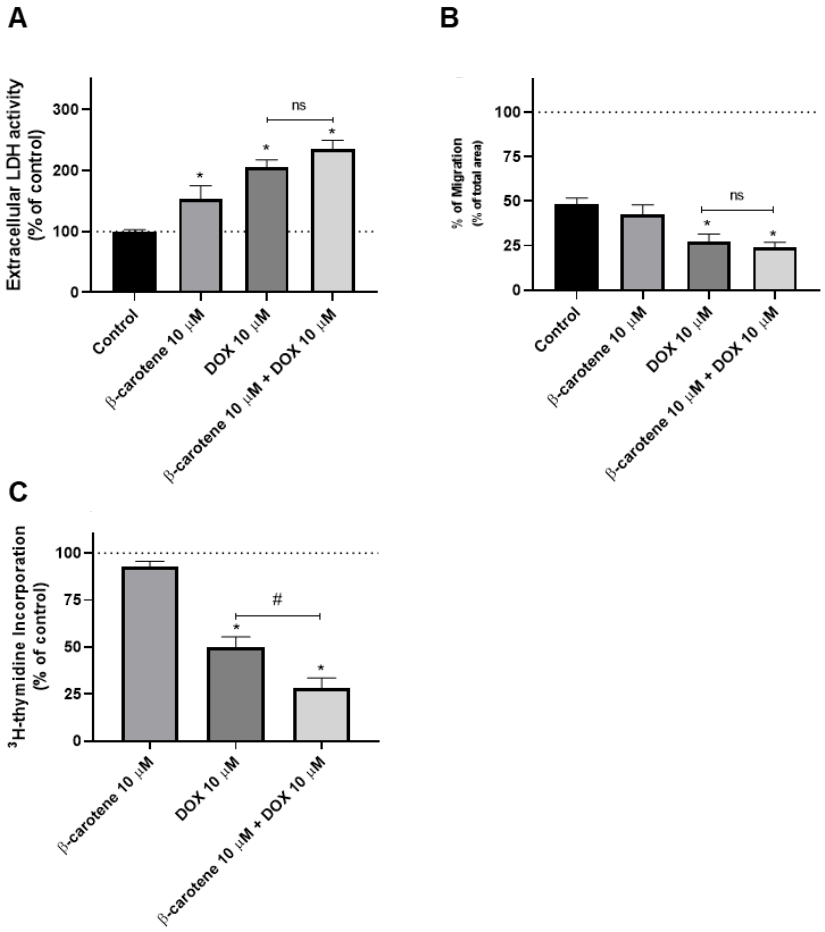

*Figure 21 - Effect of a 24 h exposure to β-carotene and/or doxorubicin on MDA-MB-231 cell viability, proliferation and migration rates. (A) Effect of β- carotene and doxorubicin on cell viability (n=9). Results are presented as LDH activity (% of control). (B) Effect of β-carotene and doxorubicin on cell migration rates. Results are presented as % of total area (n=9). (C) Effect of β-carotene and doxorubicin on proliferation rates. Results are presented as ^{3}H-thymidine incorporation (µCi/mg protein) (% of control). (n=9). Shown are arithmetic means ± SEM. *Significantly different from control (P < 0.05).*

DISCUSSION

Breast cancer is a heterogeneous disease. The insensitivity of TNBC to antihormonal treatments is an obstacle to treatment of this cancer, and consequently a high mortality rate is still observed. An alternative that is being developed is the potential use of isolated dietary compounds, mainly, carotenoids, on cancer treatment. Carotenoids reveal a protective effect against cancer, interfering with oxidative stress levels.

Before use of carotenoids as chemotherapeutic agents, a better characterization of the mechanisms that confer antitumoral effects to these compounds is necessary. An antitumoral effect of some carotenoids on breast cancer cells was described in previous studies, using MDA-MB-231 and MCF-7 cell lines. More specifically, natural and synthetic β-carotene induced apoptosis of MDA-MB-231 cells [38]. Moreover, LEG (20% lycopene) induced cell cycle arrest, caused fragmentation of DNA, inhibited cell proliferation, and caused morphological changes in MCF-7 cells [38]. Also on MCF-7 cells, crocin showed a significant antiproliferative and pro-apoptotic (via the mitochondrial pathway) effect [38, 39]. Crocin was also described as having an effective anticancer activity and anti-oxidant effect [39]. Additionally, fucoxanthin reduced the viability and induced apoptosis of MCF-7 and MDA-MB-231 cells [38]. Relatively to astaxanthin, this carotenoid significantly decreased cell viability and increased apoptosis in two distinct breast cancer cell lines (T-47D and MDA-MB-231) [40]. Based on these previous data, we selected the carotenoids β-carotene, crocin, astaxanthin and fucoxanthin to evaluate their effect on cell growth, viability, proliferation and migration of MDA-MB-231 cell line.

In relation to culture growth, astaxanthin and fucoxanthin had no significant effect on culture growth. In another cancer cell line (the HCT-116 colon cell line), the astaxanthin-rich alga *Haematococcus pluvialis* (5–25 μg/ml) inhibited cell growth in a dose- and time-dependent manner [41]. Moreover, fucoxanthin was found to induce growth arrest at G0/G1 phase in the human bladder cancer T24 cell line [42]. β-carotene (10-100-500 μM) and crocin (1-100 μM) decreased culture growth. These results are in agreement with previous studies where β-carotene (1 μM) inhibited the activation of growth signalling proteins, decreasing culture growth [38]. The results obtained with crocin are also in agreement with a previous study, where crocin inhibited prostate cancer cell invasion, by the modulation of metalloproteinases [39].

When cell viability was evaluated, fucoxanthin showed no statistically significant effect. In a previous report, fucoxanthin was shown to induce apoptosis in the MDA-MB-231 cell line [38]. β-carotene (500 μM) decreased cell viability, which is in agreement with a study that described that natural and synthetic β-carotene induced apoptosis of MDA-MB-231 cell line [38].

Crocin (10-100-500 µM) and astaxanthin (500 µM) increased cell viability. Contrary results were described in a previous study, where crocin induced apoptosis of MCF-7 cells and astaxanthin decreased MDA-MB-231 cell viability [38].

When evaluating cell proliferation, β-carotene decreased cell proliferation (1-10-100-500 µM) but no concentration-dependency was observed. These results are in agreement with data from another work, where β-carotene has an antiproliferative effect even at a lower concentration (1 µM) [31]. Crocin (10-500 µM) and astaxanthin (10-100-500 µM) also decreased cell proliferation, which is also in agreement with previous works, where crocin was found to inhibit MCF-7 cell proliferation [38] and where astaxanthin was described to inhibit proliferation of human gastric cancer cell lines, by interrupting cell cycle progression [43]. Fucoxanthin (1-10 µM) increased cell proliferation, but no concentration-dependency was observed. These data are not in agreement with Déléris et al., that described that fucoxanthin reduced the proliferation of the B16F10 melanoma cell line by inducing cell cycle arrest at the G0/G1 phase [44].

In relation to cell migration, astaxanthin and fucoxanthin showed no statistically significant effects. In the literature, previous works described that astaxanthin decreases MDA-MB-231 and MCF-7 cell migration [45] and that fucoxanthin suppressed B16-F10 melanoma cells migration [46]. In the present work, β-carotene (500 µM) and crocin (10 – 500 µM) decreased cell migration. These data are in agreement with previous studies, where migratory capabilities of SK-N-BE (2)C neuroblastoma cells were attenuated when β-carotene was applied [47] and where an anti-migratory effect of crocin was observed with AGS and HGC-27 gastric cancer cell lines [48].

Based on the results obtained, we selected the carotenoid β-carotene as the most interesting one, because it decreases the viability and inhibits growth, proliferation and migration of MDA-MB-231 cells, thus revealing an antitumor effect. So, further assays were made to better characterize its effect. As cancer cells have high energy requirements (glutamine, glucose and folic acid), in a second moment of this study, we decide to assess the effect of β-carotene in ^{3}H-GLN, ^{3}H-DG and ^{3}H-FA uptake.

The main glucose transporter GLUT1 is highly expressed in MDA-MB-231 and MCF-7 breast cancer cells [21, 22], due to high glucose requirements. We verified that β-carotene (500 µM) increased ^{3}H-DG uptake. This increase in ^{3}H-DG uptake associated with a decrease in the amount of viable cells may be due to a not well understood mechanism similar to what happens in small cell lung cancer cells, where combinations of metformin and insulin, pioglitazone and insulin and DCA (dichloroacetate) and insulin resulted in an increase in glucose uptake combined with increased apoptosis [49].

The main transporter for glutamine is ASCT2, a Na^+-coupled transporter and a target of the oncogene c-Myc, which by activating the glutamine transporter ASCT2, promotes glutamine uptake [13, 16]. LAT1 is a transporter (Na^+ -independent) also used by cancer cells for ^{3}H-GLN uptake, and is overexpressed in solid tumours (breast, prostate and lung cancer) [50]. In this study, the experiments were performed in both presence or absence of Na^+, to evaluate the effect of β-carotene on some transporters, including ASCT2 and LAT1. In the presence of Na^+, no significantly differences were observed on ^{3}H-GLN uptake. In the absence of Na^+, a decrease of ^{3}H-GLN uptake was observed with the lowest concentration of β-carotene (1 µM), that can be due to an inhibitory effect on LAT1 transporter. However, it is important to mention that this effect is most probably not relevant, because ^{3}H-GLN uptake is mainly Na^+-dependent.

The time-course of ^{3}H-FA accumulation by MDA-MB-231 cells revealed that its uptake is time-dependent, but no pH-dependency was observed. Unexpectedly, β-carotene did not have a significant effect on ^{3}H-folic acid uptake at pH 7.5 (physiological pH). On a previous work using MCF7 cells, a luminal breast cancer cell line, ^{3}H-FA was accumulated in a time-dependent manner at pH=7.5, whereas at pH 5.5 ^{3}H-FA uptake was almost undetectable and time-independent [51].

Carotenoids can decrease the oncogenic progress through the modulation of oxidative stress, cell cycle progression, apoptosis, angiogenesis, and metastasis [30]. Based on this, the next step of this work was to characterize the antitumor effect of β-carotene (10 µM), by evaluating its effect on oxidative stress levels, cell morphology and cell cycle distribution, by determining which intracellular signalling pathways are involved on its antitumor effects and by determining its effect when combined with doxorubicin. Moreover, the effect of β-carotene (10 µM) was also tested on a non-tumoral human breast epithelial cell line (MCF12-A cells), regarding cell viability, proliferation, migration and cell cycle.

Oxidative stress levels, which correlate with ROS synthesis in mitochondria, are known to interfere with the initiation, development and progression of cancer [52]. More specifically, when in excess, ROS induce cellular damage and modifications of gene expression that affect DNA repair and cell proliferation, resulting on an enhanced risk of cancer initiation [52, 53]. In relation to oxidative stress levels, β-carotene did not change oxidative stress levels, as assessed by lipid peroxidation levels and carbonyl protein content assay, revealing that the antitumor effect of this compound is not correlated with its antioxidant effect. The lack of antioxidant effect of β-carotene was not expected because of the previously demonstrated antioxidant properties of this compound, at normal oxygen pressure conditions [54]. However, when submitted to high oxygen pressures, this compound induces

loss of antioxidant properties and develops a prooxidant effect [54]. In previous works, β-carotene significantly decreased TBARS levels in buccal mucosal cells, when subjects were submitted to oral β-carotene supplementation (25 mg β-carotene/d for 7 days) [55]. However, prooxidants effects of β-carotene have also been observed, at high oxygen pressure and at high concentrations of this carotenoid [56]. TBARS and carbonyl protein content assays present low reproducibility due to the procedures used. Another method to evaluate the antioxidant activity of β-carotene is the bleaching assay. However, this method presents also low reproducibility and suffers interference from different factors, including pH and temperature [57]. So, we think that more repetitions of these technics were needed. Additionally, it is essential to optimize the protocols to improve the reproductively and sensitivity of these methods to obtain more robust results.

In relation to cell morphology, β-carotene slightly modifies this characteristic of MDA-MB-231 cells, which present an altered morphology compared to usually spindle-shaped form of non-treated cells [37]. In previous studies, the morphology of NCI-H69 small cell lung cancer cells was altered when cultured with 20 μM β-carotene. These cells had more vacuoles and lower nucleus: cytoplasm ratio than cells that were cultured without β-carotene, and that was correlated with a lower proliferation rate [58]. Also, the morphology of a cardiomyoblast cell line (H9C2) was altered when submitted to different concentrations of β-carotene [59].

The effect of β-carotene on cell cycle was also investigated, in order to determine if it interferes with the cell cycle, causing cell cycle arrest. In G1 and G2 phases, some checkpoints exist that regulate the cycle, regarding the optimum conditions for growth and division, avoiding problems with size and errors on DNA duplication [60]. Interestingly, β-carotene was found to induce an increase of cells (≈ 3,85%) retained in S phase, followed by a decrease of cells (≈12,04%) occupying the G1 phase. These results support our previous results, showing an antiproliferative effect of β-carotene in MDA-BM-231 cancer cells. In a previous study, β-carotene was described as having the capacity to inhibit MDA-MB-231 cell proliferation and promote cell cycle arrest, through the increase of cells retained in the G2/M phase, followed by a decrease of cells in G0/G1 phase [61]. The difference between our results and these results on literature could be derived from a shorter time exposure to β-carotene. From previous results on literature, cell cycle appears to be modulated by the action of β-carotene, through the action of multiple mechanisms [61]. These mechanisms are already described for some cell lines, including COLO 320 HSR, derived from human colon, where a G2/M phase arrest occurs, through downregulation of expression of cyclin A; and AtT-20 derived from mouse anterior pituitary, where S and G2/M phase arrest occurs, trough downregulation of expression of Skp2. Moreover, in the HL-90

cell line, derived from human promyelocytic leukemia, β-carotene caused G1 phase arrest [62]. So, for a better understanding of the mechanism involved in the modulation of cell cycle arrest by β-carotene, an analysis of some proteins (p53, Bax, Bcl-2, p21, p27, cyclin B, cyclin-D1 Skp2, caspase-3, caspase-7, caspase-8 and caspase-9 expression) by western blot assay, to determine which are up or downregulated, could be performed.

Carotenoids can interfere with cancer signalling pathways. So, we tested the putative involvement of different signaling pathways involved on antitumor effects of β-carotene, namely, PI3Ks, JNK, ERK/MEK1/MEK2 and p38 MAPK pathways, by testing the effect of specific inhibitors of these signaling pathways (LY294002, SP600125, PD98059, SB203580, respectively). In relation to the anti-proliferative effect of β-carotene, the results obtained allow us to conclude that activation of the JNK pathway is involved in the antiproliferative effect of β-carotene in MDA-MB-231 cells. JNK pathway, a subfamily of mitogen-activated protein kinases (MAPK), interferes with several aspects of cancer progression (namely, apoptosis, survival, proliferation, invasion and migration) in several types of cancer cells, including lung cancer, skin cancer and breast cancer [63]. Specifically on TNBC cells, the JNK pathway appears to be involved in the invasiveness of this subtype of cancer. JNK can thus be a potential target for anticancer therapy, through the development of JNK inhibitors that need to be effective and specific for specific JNK isoforms [63]. One commonly used JNK inhibitor, SP600125, is an ATP-competitive inhibitor that inhibits the phosphorylation of all JNK substrates with demonstrated effects on several cancers, including stomach and pancreatic cancer and glioblastoma [63].

Oxidative stress levels, associated with elevated ROS production, were correlated with the activation of JNK pathway [52]. Our data are not in agreement with this observation, because β-carotene did not increase oxidative stress levels but caused activation of JNK pathway, leading to cell proliferation, thus modulating cancer progression.

We also performed a comparative study with a non-tumorigenic cell line (MCF-12A), in order to determine if the effects of β-carotene are cancer cell-selective. When tested on this cell line, β-carotene (10 μM) did not affect cell viability, proliferation and migration rates and did not interfere with the cell cycle. This lack of negative effect of β-carotene on non-tumoral cell lines was previously been observed on cultured hepatocytes [64]. So, our data together with data on the literature suggest that β-carotene does not have a negative effect on non-tumorigenic cells and selectively kills cancer cells.

Due to low therapeutic options for TNBC, there is a huge necessity in finding compounds with anticancer activity against this breast cancer subtype [65]. One of these compounds is doxorubicin, an anthracycline drug, which is commonly used on chemotherapy regimens

for breast cancer [65, 66]. However, this drug presents limited applications due to its toxicity on non-cancer cells and its serious side effects on patients, including cardiotoxicity [67]. So, other compounds, which permit the selective killing of cancer cells and the reduction of side effects, need to be found. Carotenoids, used alone or in combination with doxorubicin, could eventually have the desired effect by having the capacity to kill cancer cells [65]. In our study, we evaluated cell viability, proliferation and migration rates, when MDA-MB-231 cells were submitted to a combination treatment of β-carotene with doxorubicin (10 µM), in order to determine if the combined effect of β-carotene and doxorubicin increases the anticancer effect. In relation to cell viability and migration, we observed that β-carotene (10 µM), when conjugated with doxorubicin, did not enhance the cytotoxic and the antimigratory effect of doxorubicin. This is not in agreement with data on literature, where an exposure of MDA-MB-231 cells to β-carotene (20 µM) in combination with doxorubicin (10 µM) promoted a higher decrease of cell viability, when compared to doxorubicin and β-carotene alone [65]. This difference could be related to the fact that a higher concentration of β-carotene was used in the previous report. Of importance, β-carotene (10 µM) in combination with doxorubicin potentiated the anti-proliferative effect of doxorubicin, suggesting a possible additive anti-proliferative effect. So, these results suggest that β-carotene may have a potential role as a co-adjuvant to doxorubicin for TNBC therapy, because β-carotene has cancer-selective effects and showed an additive anti-proliferative effect with doxorubicin in relation to the MDA-MB-231 cell line.

FUTURE PERSPECTIVES

In the future, we intend to test the effect of β-carotene on apoptosis and to characterize the mechanisms involved in the modulation of the cell cycle (by analysing p53, Bax, Bcl-2, p21, p27, cyclin B, cyclin-D1 Skp2, caspase-3, caspase-7, caspase-8 and caspase-9 expression), in order to better characterize its antitumoral effect. We also want to determine the effect of a longer time of exposure to β-carotene on the cell cycle, to better characterize its effects on cycle distribution and on the arrest of the cells in each phase.

Another important point would be to test and characterize the potential antitumoral effect of β-carotene in other cancer cell lines. Furthermore, we also want to test the effect of β-carotene on 3D models (spheroids), to have data in a model more similar to the *in vivo* response. Another important experiment we would like to do in the future would be to induce mammary tumours on rats and test of the effect of different amounts of β-carotene supplementation on the reduction of the tumour size, alterations on cell morphology and histological features.

CONCLUSION

With this work, we can conclude that several carotenoids interfere with MDA-MB-231 cell viability, cell culture growth, cell migration rates and cell proliferation. β-carotene decreases cell viability, proliferation, culture growth and cell migration. β-carotene significantly increases ^{3}H-L-glucose uptake by breast cancer cells and decreases Na^{+}-independent ^{3}H-L-glutamine uptake. ^{3}H-Folic Acid uptake is pH-independent and is not affected by β-carotene. So, the anti-tumoral effect of β-carotene in relation to a triple-negative breast cancer cell line is not correlated with a decrease in the cellular uptake of important nutrients (glucose, glutamine and folic acid). Moreover, it is also not correlated with an antioxidant effect, but is dependent on activation of the JNK intracellular pathway. Also, β-carotene affects cell cycle distribution of the MDA-MB-231 cell line. Importantly, β-carotene did not affect cell viability, proliferation, migration rates and cell cycle distribution of MCF12-A cells, revealing that the effect of β-carotene is cancer cell-selective. We also observed that β-carotene reveals a possible additive anti-proliferative effect in combination with doxorubicin. So β-carotene could be used as a co-adjuvant therapy with doxorubicin, increasing its anti-proliferative effect without affecting non-cancerous cells.

REFERENCES

[1] Beckmann, M. W., Niederacher, D., Schnürch, H. G., Gusterson, B. A., and Bender, H. G. (1997) Multistep carcinogenesis of breast cancer and tumour heterogeneity, *J Mol Med (Berl) 75*, 429-439.
[2] Diana, A., Franzese, E., Centonze, S., Carlino, F., Della Corte, C. M., Ventriglia, J., Petrillo, A., De Vita, F., Alfano, R., Ciardiello, F., and Orditura, M. (2018) Triple-Negative Breast Cancers: Systematic Review of the Literature on Molecular and Clinical Features with a Focus on Treatment with Innovative Drugs, *Curr Oncol Rep 20*, 76.
[3] Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., and Bray, F. (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA Cancer J Clin*.
[4] Senkus, E., Kyriakides, S., Penault-Llorca, F., Poortmans, P., Thompson, A., Zackrisson, S., Cardoso, F., and Group, E. G. W. (2013) Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up, *Ann Oncol 24 Suppl 6*, vi7-23.
[5] Al-Mahmood, S., Sapiezynski, J., Garbuzenko, O. B., and Minko, T. (2018) Metastatic and triple-negative breast cancer: challenges and treatment options, *Drug Deliv Transl Res 8*, 1483-1507.
[6] Alkabban, F. M., and Ferguson, T. (2021) Breast Cancer, In *StatPearls*, StatPearls Publishing.
[7] Singletary, S. E. (2003) Rating the risk factors for breast cancer, *Ann Surg 237*, 474-482.
[8] Sun, Y. S., Zhao, Z., Yang, Z. N., Xu, F., Lu, H. J., Zhu, Z. Y., Shi, W., Jiang, J., Yao, P. P., and Zhu, H. P. (2017) Risk Factors and Preventions of Breast Cancer, *Int J Biol Sci 13*, 1387-1397.
[9] Mohammed, F., Rashid-Doubell, F., Taha, S., Cassidy, S., and Fredericks, S. (2020) Effects of curcumin complexes on MDA-MB-231 breast cancer cell proliferation, *International Journal of Oncology 57*, 445-455.
[10] Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation, *Cell 144*, 646-674.
[11] Barbosa, A. M., and Martel, F. (2020) Targeting Glucose Transporters for Breast Cancer Therapy: The Effect of Natural and Synthetic Compounds, *Cancers (Basel) 12*.
[12] Cazzaniga, M., and Bonanni, B. (2015) Breast Cancer Metabolism and Mitochondrial Activity: The Possibility of Chemoprevention with Metformin, *Biomed Res Int 2015*, 972193.
[13] Cha, Y. J., Kim, E. S., and Koo, J. S. (2018) Amino Acid Transporters and Glutamine Metabolism in Breast Cancer, *Int J Mol Sci 19*.
[14] Still, E. R., and Yuneva, M. O. (2017) Hopefully devoted to Q: targeting glutamine addiction in cancer, *Br J Cancer 116*, 1375-1381.
[15] Scalise, M., Pochini, L., Galluccio, M., Console, L., and Indiveri, C. (2017) Glutamine Transport and Mitochondrial Metabolism in Cancer Cell Growth, *Frontiers in Oncology 7*.
[16] Dong, Y., Tu, R., Liu, H., and Qing, G. (2020) Regulation of cancer cell metabolism: oncogenic MYC in the driver's seat, *Signal Transduct Target Ther 5*, 124.
[17] Liu, Y., Zhao, T., Li, Z., Wang, L., Yuan, S., and Sun, L. (2018) The role of ASCT2 in cancer: A review, *Eur J Pharmacol 837*, 81-87.
[18] Augustin, R. (2010) The protein family of glucose transport facilitators: It's not only about glucose after all, *IUBMB Life 62*, 315-333.
[19] Martel, F., Guedes, M., and Keating, E. (2016) Effect of polyphenols on glucose and lactate transport by breast cancer cells, *Breast Cancer Res Treat 157*, 1-11.
[20] Carvalho, K. C., Cunha, I. W., Rocha, R. M., Ayala, F. R., Cajaíba, M. M., Begnami, M. D., Vilela, R. S., Paiva, G. R., Andrade, R. G., and Soares, F. A. (2011) GLUT1 expression in malignant tumors and its use as an immunodiagnostic marker, *Clinics (Sao Paulo) 66*, 965-972.
[21] Macheda, M. L., Rogers, S., and Best, J. D. (2005) Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer, *J Cell Physiol 202*, 654-662.
[22] Furuta, E., Okuda, H., Kobayashi, A., and Watabe, K. (2010) Metabolic genes in cancer: their roles in tumor progression and clinical implications, *Biochim Biophys Acta 1805*, 141-152.
[23] Pragallapati, S., and Manyam, R. (2019) Glucose transporter 1 in health and disease, *J Oral Maxillofac Pathol 23*, 443-449.

[24] Tomita, L. Y. (2016) Folate and Cancer, *Journal of Inborn Errors of Metabolism and Screening 4*.
[25] Rosenzweig, A., Blenis, J., and Gomes, A. P. (2018) Beyond the Warburg Effect: How Do Cancer Cells Regulate One-Carbon Metabolism?, *Frontiers in Cell and Developmental Biology 6*.
[26] Koseki, J., Konno, M., Asai, A., Colvin, H., Kawamoto, K., Nishida, N., Sakai, D., Kudo, T., Satoh, T., Doki, Y., Mori, M., and Ishii, H. (2018) Enzymes of the one-carbon folate metabolism as anticancer targets predicted by survival rate analysis, *Sci Rep 8*, 303-303.
[27] Yang, M., and Vousden, K. H. (2016) Serine and one-carbon metabolism in cancer, *Nature Reviews Cancer 16*, 650-662.
[28] Gonen, N., and Assaraf, Y. G. (2012) Antifolates in cancer therapy: Structure, activity and mechanisms of drug resistance, *Drug Resistance Updates 15*, 183-210.
[29] Hazlehurst, L., and Hacker, M. (2009) Chapter 15 - Drug Resistance, In *Pharmacology* (Hacker, M., Messer, W., and Bachmann, K., Eds.), pp 371-385, Academic Press, San Diego.
[30] Saini, R. K., Keum, Y. S., Daglia, M., and Rengasamy, K. R. (2020) Dietary carotenoids in cancer chemoprevention and chemotherapy: A review of emerging evidence, *Pharmacol Res 157*, 104830.
[31] Sowmya Shree, G., Yogendra Prasad, K., Arpitha, H. S., Deepika, U. R., Nawneet Kumar, K., Mondal, P., and Ganesan, P. (2017) β-carotene at physiologically attainable concentration induces apoptosis and down-regulates cell survival and antioxidant markers in human breast cancer (MCF-7) cells, *Mol Cell Biochem 436*, 1-12.
[32] Cui, Y., Lu, Z., Bai, L., Shi, Z., Zhao, W.-e., and Zhao, B. (2007) β-Carotene induces apoptosis and up-regulates peroxisome proliferator-activated receptor γ expression and reactive oxygen species production in MCF-7 cancer cells, *European Journal of Cancer 43*, 2590-2601.
[33] Nogueira, J., Soares, S. F., Amorim, C. O., Amaral, J. S., Silva, C., Martel, F., Trindade, T., and Daniel-da-Silva, A. L. (2020) Magnetic Driven Nanocarriers for pH-Responsive Doxorubicin Release in Cancer Therapy, *Molecules 25*, 333.
[34] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry 72*, 248-254.
[35] Silva, C., Andrade, N., Rodrigues, I., Ferreira, A. C., Soares, M. L., and Martel, F. (2021) The pro-proliferative effect of interferon-γ in breast cancer cell lines is dependent on stimulation of ASCT2-mediated glutamine cellular uptake, *Life Sci 286*, 120054.
[36] Silva, C., Andrade, N., Guimarães, J. T., Patrício, E., and Martel, F. (2021) The in vitro effect of the diabetes-associated markers insulin, leptin and oxidative stress on cellular characteristics promoting breast cancer progression is GLUT1-dependent, *European Journal of Pharmacology 898*, 173980.
[37] Ivers, L. P., Cummings, B., Owolabi, F., Welzel, K., Klinger, R., Saitoh, S., O'Connor, D., Fujita, Y., Scholz, D., and Itasaki, N. (2014) Dynamic and influential interaction of cancer cells with normal epithelial cells in 3D culture, *Cancer Cell Int 14*, 108.
[38] Maghsoudi, S., Taghavi Shahraki, B., Rabiee, N., Fatahi, Y., Bagherzadeh, M., Dinarvand, R., Ahmadi, S., Rabiee, M., Tahriri, M., Hamblin, M. R., Tayebi, L., and Webster, T. J. (2021) The colorful world of carotenoids: a profound insight on therapeutics and recent trends in nano delivery systems, *Crit Rev Food Sci Nutr*, 1-40.
[39] Hoshyar, R., and Mollaei, H. (2017) A comprehensive review on anticancer mechanisms of the main carotenoid of saffron, crocin, *J Pharm Pharmacol 69*, 1419-1427.
[40] Karimian, A., Bahadori, M. h., Hajizadeh Moghaddam, A., and Mir Mohammadrezaei, F. (2017) Effect of Astaxanthin on cell viability in T-47D and MDA-MB-231 Breast Cancer Cell Lines, *mcijournal 1*, 0-0.
[41] Palozza, P., Torelli, C., Boninsegna, A., Simone, R., Catalano, A., Mele, M. C., and Picci, N. (2009) Growth-inhibitory effects of the astaxanthin-rich alga Haematococcus pluvialis in human colon cancer cells, *Cancer Letters 283*, 108-117.

[42] Wang, L., Zeng, Y., Liu, Y., Hu, X., Li, S., Wang, Y., Li, L., Lei, Z., and Zhang, Z. (2014) Fucoxanthin induces growth arrest and apoptosis in human bladder cancer T24 cells by up-regulation of p21 and down-regulation of mortalin, *Acta Biochim Biophys Sin (Shanghai) 46*, 877-884.
[43] Kim, J. H., Park, J. J., Lee, B. J., Joo, M. K., Chun, H. J., Lee, S. W., and Bak, Y. T. (2016) Astaxanthin Inhibits Proliferation of Human Gastric Cancer Cell Lines by Interrupting Cell Cycle Progression, *Gut Liver 10*, 369-374.
[44] Déléris, P., Nazih, H., and Bard, J. M. (2016) Chapter 10 - Seaweeds in Human Health, In *Seaweed in Health and Disease Prevention* (Fleurence, J., and Levine, I., Eds.), pp 319-367, Academic Press, San Diego.
[45] McCall, B., McPartland, C. K., Moore, R., Frank-Kamenetskii, A., and Booth, B. W. (2018) Effects of Astaxanthin on the Proliferation and Migration of Breast Cancer Cells In Vitro, *Antioxidants 7*, 135.
[46] 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., and Ha, K. T. (2013) Marine algal fucoxanthin inhibits the metastatic potential of cancer cells, *Biochem Biophys Res Commun 439*, 580-585.
[47] Kim, Y.-S., Lee, H.-A., and Kim, Y. (2013) The effect of beta-carotene on migration and invasion in SK-N-BE(2)C neuroblastoma cells, *The FASEB Journal 27*, 639.616-639.616.
[48] Zhou, Y., Xu, Q., Shang, J., Lu, L., and Chen, G. (2019) Crocin inhibits the migration, invasion, and epithelial-mesenchymal transition of gastric cancer cells via miR-320/KLF5/HIF-1α signaling, *J Cell Physiol 234*, 17876-17885.
[49] Domvri, K., Zogas, N., Zarogoulidis, P., Petanidis, S., Porpodis, K., Kioseoglou, E., and Zarogoulidis, K. (2016) AB060. Could glucose uptake ability enhanced by drugs result in antitumor effect in small cell lung cancer?, *Annals of Translational Medicine 4*.
[50] Häfliger, P., and Charles, R.-P. (2019) The L-Type Amino Acid Transporter LAT1-An Emerging Target in Cancer, *International journal of molecular sciences 20*, 2428.
[51] Guedes, M., Araujo, J. R., Correia-Branco, A., Gregorio, I., Martel, F., and Keating, E. (2016) Modulation of the uptake of critical nutrients by breast cancer cells by lactate: Impact on cell survival, proliferation and migration, *Exp Cell Res 341*, 111-122.
[52] Arfin, S., Jha, N. K., Jha, S. K., Kesari, K. K., Ruokolainen, J., Roychoudhury, S., Rathi, B., and Kumar, D. (2021) Oxidative Stress in Cancer Cell Metabolism, *Antioxidants (Basel) 10*, 642.
[53] Klaunig, J. E. (2018) Oxidative Stress and Cancer, *Curr Pharm Des 24*, 4771-4778.
[54] Burton, G. W., and Ingold, K. U. (1984) β-Carotene: an Unusual Type of Lipid Antioxidant, *Science 224*, 569-573.
[55] Erhardt, J. G., Mack, H., Sobeck, U., and Biesalski, H. K. (2002) β-Carotene and α-tocopherol concentration and antioxidant status in buccal mucosal cells and plasma after oral supplementation, *British Journal of Nutrition 87*, 471-475.
[56] Palozza, P., Serini, S., Di Nicuolo, F., Piccioni, E., and Calviello, G. (2003) Prooxidant effects of β-carotene in cultured cells, *Molecular Aspects of Medicine 24*, 353-362.
[57] Prieto, M. A., Rodríguez-Amado, I., Vázquez, J. A., and Murado, M. A. (2012) β-Carotene Assay Revisited. Application To Characterize and Quantify Antioxidant and Prooxidant Activities in a Microplate, *Journal of Agricultural and Food Chemistry 60*, 8983-8993.
[58] Prakash, P., Manfredi, T. G., Jackson, C. L., and Gerber, L. E. (2002) β-Carotene Alters the Morphology of NCI-H69 Small Cell Lung Cancer Cells, *The Journal of Nutrition 132*, 121-124.
[59] Lesmana, R., Yusuf, I., Goenawan, H., Achadiyani, A., Khairani, A. F., Fatimah, S., and Supratman, U. (2020) Low Dose of β-Carotene Regulates Inflammation, Reduces Caspase Signaling, and Correlates with Autophagy Activation in Cardiomyoblast Cell Lines, *Medical Science Monitor Basic Research 26*.
[60] Tan, E. P., Duncan, F. E., and Slawson, C. (2017) The sweet side of the cell cycle, *Biochem Soc Trans 45*, 313-322.
[61] GLORIA, N. F., SOARES, N., BRAND, C., OLIVEIRA, F. L., BOROJEVIC, R., and TEODORO, A. J. (2014) Lycopene and Beta-carotene Induce Cell-Cycle Arrest and Apoptosis in Human Breast Cancer Cell Lines, *Anticancer Research 34*, 1377-1386.

[62] Niranjana, R., Gayathri, R., Nimish Mol, S., Sugawara, T., Hirata, T., Miyashita, K., and Ganesan, P. (2015) Carotenoids modulate the hallmarks of cancer cells, *Journal of Functional Foods 18*, 968-985.

[63] Wu, Q., Wu, W., Jacevic, V., Franca, T. C. C., Wang, X., and Kuca, K. (2020) Selective inhibitors for JNK signalling: a potential targeted therapy in cancer, *J Enzyme Inhib Med Chem 35*, 574-583.

[64] Yang, S.-C., Huang, C.-C., Chu, J.-S., and Chen, J.-R. (2004) Effects of β-carotene on cell viability and antioxidant status of hepatocytes from chronically ethanol-fed rats, *British Journal of Nutrition 92*, 209-215.

[65] Vijay, K., Sowmya, P. R.-R., Arathi, B. P., Shilpa, S., Shwetha, H. J., Raju, M., Baskaran, V., and Lakshminarayana, R. (2018) Low-dose doxorubicin with carotenoids selectively alters redox status and upregulates oxidative stress-mediated apoptosis in breast cancer cells, *Food and Chemical Toxicology 118*, 675-690.

[66] Liu, M., Yin, H., Qian, X., Dong, J., Qian, Z., and Miao, J. (2016) Xanthohumol, a Prenylated Chalcone from Hops, Inhibits the Viability and Stemness of Doxorubicin-Resistant MCF-7/ADR Cells, *Molecules 22*.

[67] Alkaraki, A., Alshaer, W., Wehaibi, S., Gharaibeh, L., Abuarqoub, D., Alqudah, D. A., Al-Azzawi, H., Zureigat, H., Souleiman, M., and Awidi, A. (2020) Enhancing chemosensitivity of wild-type and drug-resistant MDA-MB-231 triple-negative breast cancer cell line to doxorubicin by silencing of STAT 3, Notch-1, and β-catenin genes, *Breast Cancer 27*, 989-998.

SUPPLEMENTARY MATERIAL

Manuscript, (2022)

Effect of Carotenoids on Nutrient Uptake by MDA-MB 231 cells

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Data available on request from the authors: the data that support the findings of this study are available from the corresponding author, F. Martel, upon reasonable request.

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Abstract

Breast cancer is a major cause of cancer-related mortality in women worldwide. Triple-negative breast cancer presents an aggressive behavior and a poor response to therapeutic. Cancer progression is associated with the reprogramming of metabolic pathways for glutamine, glucose and folic acid. In this study, we characterized the antitumor effect of carotenoids on a triple-negative human breast cancer cell line (MDA-MB-231 cell line), and investigated interference with nutrient cellular uptake as a contributing mechanism. Of the four tested carotenoids (β-carotene, crocin, fucoxanthin, astaxanthin), β-carotene presents the most interesting antitumoral effect, by reducing cell proliferation, culture growth, migration and viability and interfering with cell cycle (S phase arrest). β-carotene significantly increased ^{3}H-deoxy-D-glucose uptake, did not affect ^{3}H-glutamine uptake and did not affect ^{3}H-folic acid uptake. It did also not interfere with oxidative stress levels. The anti-proliferative effect of β-carotene involves activation of the JNK intracellular pathway, and it was able to enhance the anti-proliferative effect of doxorubicin. Importantly, β-carotene did not affect cell viability, proliferation, cell cycle and migration rates of MCF-12A cells, a non-tumoral human epithelial cell line. In conclusion, β-carotene presents potential as co-adjuvant to doxorubicin for triple-negative breast cancer treatment.

Keywords: MDA-MB-231, MCF12-A, carotenoids, β-carotene, doxorubicin

Introduction

Breast cancer became in 2020 the most incident cancer worldwide and the third major cause of death in Europe (1, 2). Breast cancer is a heterogeneous disease and the triple negative (TNBC) or basal type, characterized by the absence of progesterone,

estrogen and HER2 (human epidermal growth factor receptor 2) (1, 3) presents limited treatments, due to the absence of targeted therapies (3).

For cancer development, high energy and nutrient levels are required, including glucose, glutamine and folic acid (4). To accomplish these needs, cancer cells exhibit a reprogramming of some metabolic pathways, also designed deregulation of cellular energetics, which was recently included in the hallmarks of cancer cells (5).

Glutamine is a nutrient essential for cancer proliferation and progression, as a source of nitrogen, which is essential to synthesize non-essential amino acids, contributing to redox balance, through glutathione synthesis, and as a source of ATP (6, 7). TNBC have an increased need of glutamine - "glutamine addiction" – that could be a potential therapeutic target (6). This increased need of glutamine requires glutamine cellular transporters, including the ASCT2 (alanine, serine, cysteine transporter 2) and LAT1, that can be a potential target for chemotherapeutic drugs (7, 8). ASCT2 is overexpressed in some cancers and associated with poor prognosis, being used as a prognostic marker (9). The glucose cellular reprogramming on cancer cells includes a metabolic shift from mitochondrial oxidative phosphorylation to aerobic glycolysis ("Warburg effect"), associated with high lactate production and increased glycolytic rates (5). The glucose transporter GLUT1 is highly expressed in various types of tumors, including breast and ovarian cancer, being associated with an aggressive behavior and proliferation, and consequently poor prognosis and reduced survival rates (10-12). Moreover, one carbon metabolism, which is dependent on folates, is essential for cancer cells, as it is involved in DNA synthesis and methylation process, in holding DNA integrity, gene expression and cellular homeostasis and thus in supporting the growth and proliferation of the cells (13-15). In accordance with the importance of folates for cancer

cells, targeting folate-mediated one-carbon metabolism is a strategy used in cancer therapy, through antifolates (eg, methotrexate, ralitrexed and pemetrexed) (14, 16).

Carotenoids are tetraterpenoids with antioxidant properties and anticancer activity (chemopreventive and chemotherapeutic capacity) (17). Carotenoids have a significant antitumoral effect mediated by a wide range of molecular mechanisms modulating oxidative stress and redox balance, cellular signaling proteins, transcription factors, apoptosis, cell cycle progression and proliferation, angiogenesis, metastasis, gap junction intercellular communication and multidrug resistance (17). For instance, β-carotene was found to induce apoptosis, increase caspase 3-activity, and decrease the expression of anti-apoptotic (Bcl-2 and PARP) and survival (NF-kB) proteins in MCF-7 breast cancer cells (18). They can act on multiple cancer cell intracellular signaling pathways, including PI3K/AKT/mTOR, p38, ERK (extracellular signal-regulated kinase) and JNK (c-Jun N-terminal kinases) (17).

The aim of this work is to evaluate the potential antitumor effect of carotenoids (β-carotene, crocin, fucoxanthin and astaxanthin) on TNBC cells and to investigate if interference with glucose, glutamine or folic acid cellular uptake can contribute to their antitumoral effect.

Material and Methods

Materials

^{3}H-Deoxy-D-glucose (2-[1,2-^{3}H(N)]-deoxy-D-glucose: specific activity 60 Ci/mmol); ^{3}H-L-glutamine, (L-[2,3,4-^{3}H]; specific activity 60 Ci/mmol) (American Radiolabeled Chemicals Inc., St Louis, MO, USA); ^{3}H-folic acid [3,5,7,9-3H] (specific activity 40 Ci/mmol) (American Radiolabeled Chemicals Inc., St Louis, MO, USA); ^{3}H-thymidine ([methyl-^{3}H]-thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH,

Freiburg, Germany); heat-inactivated horse serum (Gibco, Life Technologies Corporation, CA, USA); D-glucose; hydrochloric acid (HCl); triton X-100; trichloroacetic acid (TCA) (Merck, Darmstadt, Germany); acetic acid (Scientific Laboratory Supplies, Wilford, United Kingdom); DMEM/F12 medium (1:1) (catalogue P04-41150; PAN-Biotech, Aidenbach, Germany); antibiotic/antimycotic solution (100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B); bovine serum albumin (BSA); fetal bovine serum (FBS); astaxanthin; β-carotene; cholera toxin; crocin; fucoxanthin; reduced nicotinamide adenine dinucleotide (NADH); RPMI 1640 medium; sodium hydroxide (NaOH); sodium pyruvate; sulforhodamine B (SRB); tris-(tris(hydroxymethyl)-aminomethane hydrochloride); trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Sigma, St. Louis, MO, USA). Doxorubicin hydrochloride (≥ 95%; DOX HCl); LY294002; SP600125; PD98059; SB203580; Dinitrophenylhydrazine (DNPH); guanidine (Sigma); PI (propridium iodide); hydroxyethylpiperazine-N0-2-ethanesulfonic acid (HEPES).

Cell Culture

The MDA-MB-231 cell line (a triple negative human breast epithelial cancer cell line) and the MCF-12A cell line (a non-tumorigenic human breast epithelial) were obtained from the American Type Culture Collection (ATCC) and used between passage number 53 to 78 (MDA-MB-231) and 33 to 37 (MCF-12A). MDA-MB-231 cells were grown in RPMI 1640 medium (catalogue #R6504, Sigma, St Louis, MI, USA) supplemented with 2 mM L-glutamine, 10 mM sodium bicarbonate, 15% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic/antimycotic solution. MCF-12A cells were maintained in DMEM/F12 medium (1:1) (catalogue P04-41150; PAN-Biotech, Aidenbach, Germany) supplemented with 20 ng/ml human epidermal growth factor, 100

ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone, 5% heat-inactivated horse serum and 1% antibiotic/antimycotic solution.

Cells were maintained in a humidified atmosphere of 95% air and 5% CO2. Culture medium was changed every 3-4 days and the culture was split every 7 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 4 min, 37°C), split 1:3 ratio and sub-cultured in plastic culture dishes (21 cm2; diameter 60 mm; TPP®, Trasadingen, Switzerland).

For determination of cell viability, proliferation, migration and culture growth, cells were seeded on 24-well plastic cell culture dishes (2 cm^2 Ø 16 mm; TPP®) and used at 90% confluence (4 days-old culture). For ^{3}H-glutamine (^{3}H-GLN), ^{3}H-deoxy-d-glucose (^{3}H-DG) and ^{3}H-folic acid (^{3}H-FA) uptake experiments, cells were seeded similarly and used at 100% confluence (5-days old culture). For protein carbonyl content and TBARS assay, cells were seeded on 12-well plastic cell culture dishes (3.60 cm^2; TPP®) and used at 100% confluence (5-days old culture). For cell cycle analysis, cells were seeded in plastic culture dishes (21 cm^2; diameter 60 mm; TPP®, Trasadingen, Switzerland) and used at 100% confluence (5-days old culture).

Treatment of the cells

The carotenoids used in this study were: astaxanthin, β-carotene, crocin and fucoxanthin. The concentrations of carotenoids (1 μM, 10 μM, 100 μM and 500 μM) and doxorubicin (10 μM) to be tested were chosen based on previous works of our group (19). MDA-MB-231 and MCF-12A cells were treated for 24h with carotenoids and/or other compounds in serum-free culture medium. Astaxanthin and β-carotene were dissolved in DMSO, crocin and doxorubicin were dissolved in H_2O and fucoxanthin in ethanol. For

controls, the solvent of each compound was used. The final concentration of these solvents in culture medium was 1% (v/v).

Incorporation of ^{3}H-thymidine assay - Determination of cell proliferation rates

Cellular proliferation rates were determined by the incorporation of ^{3}H-thymidine, that quantifies DNA synthesis. For this, MDA-MB-231 and MCF-12A cells were exposed for 24 h to carotenoids or doxorubicin (or the solvents) and incubation with ^{3}H-thymidine 0.025 μCi/ml was done in the last 5h of treatment. The excess of ^{3}H-thymidine was rinsed off with 10% TCA, followed by a 20 min incubation with NaOH 1 M (280 μl/well). Before the addition of scintillation fluid, the lysate was neutralized with 5 mM HCl. The radioactivity of the samples was then quantified by liquid scintillometry (LKB Wallac Liquid Scintillation Counter). Cellular DNA synthesis rate was expressed as incorporation of ^{3}H-thymidine (μCi/mg protein).

SRB assay - Determination of culture growth

Sulforhodamine B has the capacity to electrostatically bind to basic amino acids of cell culture proteins previously fixated with TCA (trichloroacetic acid). It measures whole-culture protein content as an index of culture growth. After the 24 h treatment with carotenoids (or their solvents), culture medium was replaced with 500 μL PBS 1x and 62.5 μL of ice cold 50% (w/v) TCA was added to each well to fix cells, for 1 h at 4°C in the dark. Then, cells were washed with tap water to remove excess TCA, air dried, and then stained for 15 min with 125 μL 0.4% (w/v) SRB dissolved in 1% acetic acid per well. After SRB removal, cells were rinsed with 1% acetic acid to remove residual die, and then air dried again. The bound die was solubilized with 375 μL of 10 mM Tris-NaOH solution (pH=10.5). To obtain absorbance (540 nm) values below 0.7, the contents

of each well were diluted and placed in a 96-well plaque where the absorbance was measured using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Extracellular LDH assay - Determination of cell viability

Cell death is correlated to leakage of lactate dehydrogenase (LDH) to the extracellular medium and measurement of LDH activity, through the measurement of the decrease in absorbance of NADH during the reduction of pyruvate to lactate, can be used to evaluate cell viability. MDA-MB-231 and MCF12-A cells were submitted to a 24 h treatment with carotenoids and/or other compounds (or their solvents). After this period, the extracellular medium was removed and 50 µL of extracellular medium was added to 250 µL of a mixture of phosphate/pyruvate (50 mM/0.63 mM) solution and NADH (11.3 mM) solution. Absorbance values were measured in a 96-well plaque at 340 nm, and the decrease in absorbance value, in this time interval, was calculated. LDH activity was expressed as the percentage of extracellular activity in relation to total cellular LDH activity, which represents 100% cell death. To determine the total activity, control cells were solubilized with 500 µL of 0.1% (v/v) Triton X-100 and incubated for 1 h at 4°C, and then the sample was processed in the same way as the others.

^{3}H-L-glutamine (^{3}H-GLN), ^{3}H-deoxy-d-glucose (^{3}H-DG) and ^{3}H-folic acid transport assays

For ^{3}H-glutamine (^{3}H-GLN) and ^{3}H-deoxy-d-glucose (^{3}H-DG) uptake measurements, the experiments were performed in GF-HBS buffer (glucose-free HEPES buffered saline) (composition, in mM: 140 NaCl, 5 KCl, 20 HEPES, 2.5 $MgSO_4$, 1 $CaCl_2$; pH 7.4). Sodium-independent ^{3}H-GLN uptake was determined by using GF-HBS buffer

in which NaCl was replaced with 140 mM LiCl. For ^{3}H-folic acid (^{3}H-FA) uptake measurement, the experiments were performed in HMBS buffer (HEPES-MES buffered saline) (composition, in mM: 140 NaCl, 5 KCl, 12.5 HEPES, 12.5 MES, 5 glucose, 1.2 $MgSO_4$, 1.2 $CaCl_2$; pH 7.5 or 5.5).

The culture medium was removed, and the cells were washed with 300 µL GF-HBS or HMBS buffer at 37°C. Then, cell monolayers were preincubated for 20 min in GF-HBS or HMBS buffer at 37°C. Uptake was then initiated by the addition of 200 µL buffer at 37°C containing 5 nM ^{3}H-GLN, 10 nM ^{3}H-DG or 20 nM ^{3}H-FA. When tested, carotenoids (or the solvents) were present during both pre-incubation and incubation periods. Incubation was stopped after 6 min, by removing the incubation medium, placing the cells on ice, and rinsing the cells with 500 µL ice-cold GF-HBS or HMBS buffer. The cells were then solubilized with 300 µL 0.1 % (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4), and placed at 4°C overnight. The radioactivity of the samples was quantified by liquid scintillometry (LKB Wallac Liquid Scintillation Counter 1209, Turku, Finland).

Determination of oxidative stress-induced cellular damage

TBARS assay - Determination of oxidative stress levels

After a 24h treatment of MDA-MB-231 cells with β-carotene (10 µM) (or its solvent), an homogenization solution (KH_2PO_4 62.5 nM, $Na_2HPO_4.2H_2O$ 50 nM, Triton X-100 0.1 %, pH 7) was added to each sample and cells were resuspended. The reaction was started by the addition of 200 µL TCA 50% to each sample, followed by a centrifugation for 2 min at 10000 rpm (4ºC). Then, 1% 2-thiobarbituric acid (TBA) was added to the supernatant and an incubation was carried out for 40 min at 95 ºC. After that, 300 µL of each sample were transferred to a 96-well culture plate, and the colored complex was quantified spectrophotometrically at 535 nm using a microplate reader

(Thermo Fisher Scientific, Waltham, Massachusetts, USA). The MDA standard curve was performed using MDA bis(dimethyl acetal), subjected to an acid hydrolysis (addition of 0.1 M HCl and incubation at 40 °C for 1 h). The results were normalized to the protein content.

Protein Carbonyl content assay

MDA-MB-231 cells were treated for 24 h with β-carotene (10 µM) (or its solvent). At the end of the treatment, the medium was removed and the cells were washed with PBS solution. The cells were then resuspended in homogenization solution and 100 µL of this solution were added to a microtube, in duplicates, and 200 µL of TCA 10 % were added. The samples were then centrifuged at 13000 rpm for 2 min at 4 °C and the supernatant was discarded. The duplicates were treated with 500 µL of DNPH (dinitrophenylhydrazine) or 500 µL HCl and rested for 1 h. After that, 500 µL of TCA 20 % at 4 °C were added, the samples were kept on ice for 15 min and then centrifuged at 13000 rpm for 2 min at 4 °C. Then, a wash with ethanol:ethyl acetate (1:1) for two times was done, with centrifugations in between at 13000 for 2 min at 4 °C. We next dissolved the sediment overnight with 500 µL of guanidine 6 M at 4 °C, followed by a centrifugation at 3000 rpm for 15 min. The absorbances were then measured with a microplate reader at 340 nM (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Cell Cycle Analysis

Changes in cell distribution across cell cycle stages were assessed by measurement of DNA content in the cells. Cells were treated for a 24 h with β-carotene (10 µM) (or DMSO). At the end of the treatment, the medium was removed and the cells were washed with PBS solution. Then, cells were removed enzymatically (0.25% trypsin–EDTA, 4 min, 37°C), resuspended in 3 mL medium without serum, and submitted to a

centrifugation (5 min, 300 g). The supernatant was removed and the pellet was re-suspended in 200 µL of 70% ethanol, and then stored at -20ºC. Next, cells were washed in PBS with 2% BSA and re-suspended in 100 µL of propidium iodide (PI) solution (PI/Rnase, Immunostep) for 15 min at room temperature. Flow cytometry analysis was performed on a BD Accuri C6 flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Results were analysed using FlowJo version 10.7.1 software (Ashland, Oregon). Results are expressed as % of total cells arrested in each phase in the cell cycle.

Protein determination

The protein content of the cell monolayers was determined as described as Bradford (20), using human serum albumin as standard.

Statistics

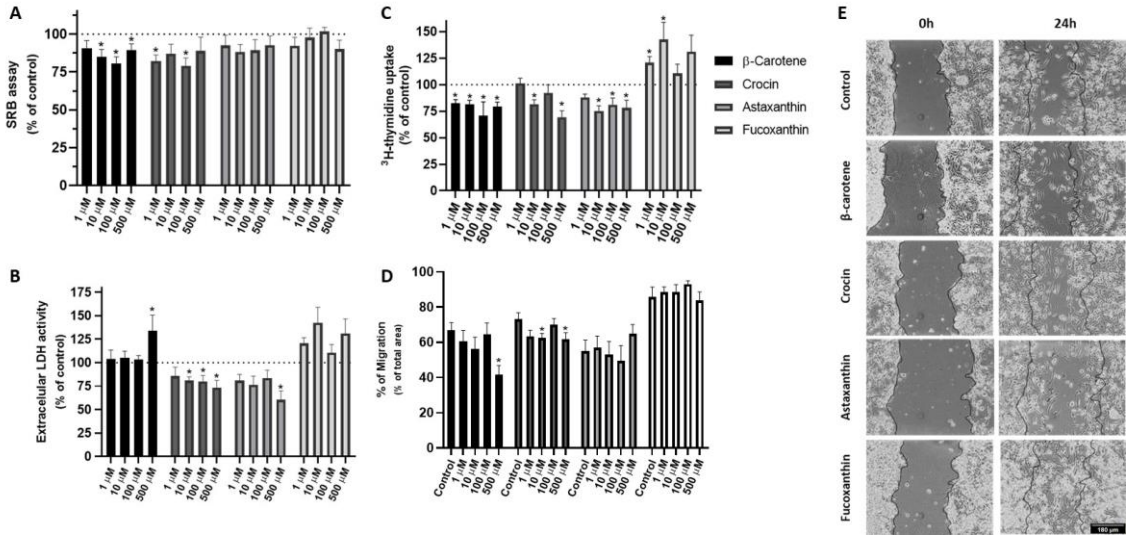
Arithmetic means are given with standard error of the mean (SEM). The value of n indicates the number of replicates of at least two different experiments. Statistical significance of the difference between three or more groups was evaluated by one-way ANOVA test, followed by the Student-Newman-Keuls test. For comparison between 2 groups, Student t test was used. Differences were considered significant when $P < 0.05$. Analyses were done using the GraphPad Prism version 9.0 software (San Diego, CA, USA).

Results

Effect of carotenoids on culture growth and cell viability, proliferation and migration ability of MDA-MB-231 cells

In the first series of experiments, the effects of four different carotenoids (β-carotene, crocin, astaxanthin and fucoxanthin) on culture growth, cell viability, cell proliferation and migration ability of MDA-MB-231 cells were evaluated. Of these, β-carotene (all tested concentrations, with no concentration-dependency observed) and crocin (1 and 100 µM) significantly decreased culture growth (Fig. 1A). Moreover, β-carotene (500 µM) decreased cell viability, and crocin (10-100-500 µM) and astaxanthin (500 µM) increased it (Fig. 1B). Regarding cell proliferation, β-carotene (all concentrations tested, with no concentration dependency observed), astaxanthin (10-500 µM) and crocin (10 and 500 µM) decreased it and, in contrast, fucoxanthin (1-10 µM) increased this parameter (Fig. 1C). Finally, β-carotene (500 µM) and crocin (10 and 500 µM) decreased cell migration (Fig. 1D). Representative images of cell migration (injury assay) are presented (Fig. 1E) **[Figure 1 near here]**

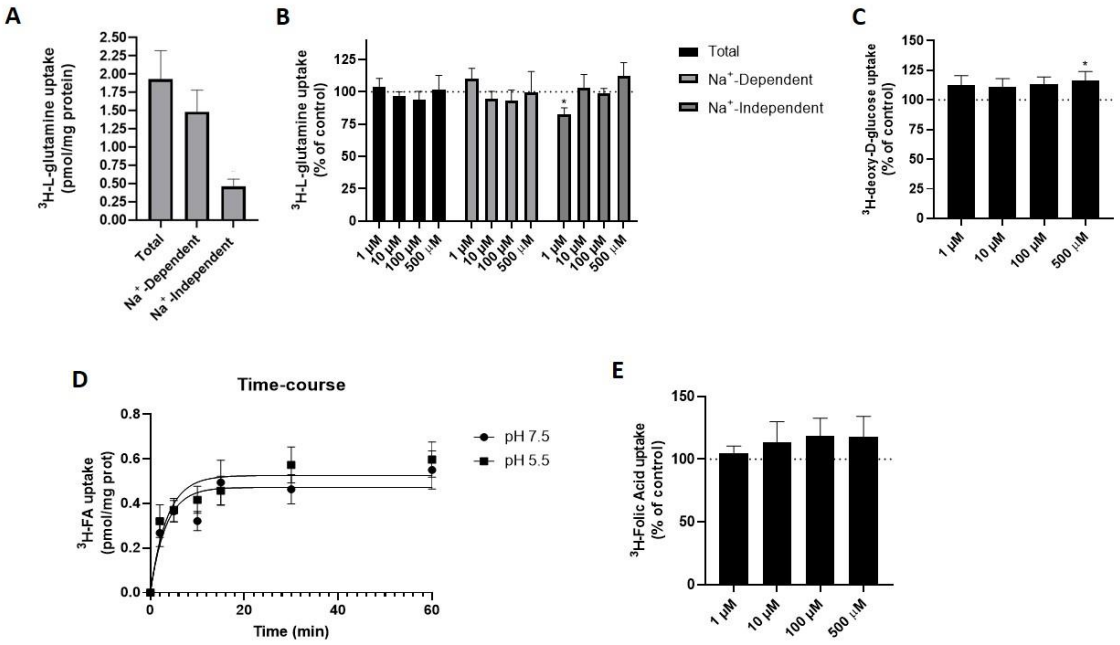
Based on these results, β-carotene was selected for further assays, because it decreases cell viability and proliferation, culture growth and migration ability of MDA-MB-231 cells, thus revealing an antitumoral effect.



Effect of β-carotene on ^{3}H-GLN, ^{3}H-DG uptake and ^{3}H-FA cellular uptake by MDA-MB-231 cells

In this series of experiments, the effects of β-carotene on the cellular uptake of ^{3}H-GLN, ^{3}H-DG and ^{3}H-FA by the MDA-MB-231 cell line were evaluated.

The MDA-MB-231 cell line was previously shown by our group to take up ^{3}H-GLN in a time-dependent manner, mainly by a Na^{+}-dependent mechanism (21), and this was confirmed in this study (Fig. 2A). In relation to total and Na^{+}-dependent ^{3}H-GLN uptake, β-carotene showed no significant effect. In contrast, β-carotene (1 μM) slightly decreased Na^{+}-independent ^{3}H-GLN uptake (Fig. 2B). The MDA-MB-231 cell line is known to take up ^{3}H-DG in a time-dependent manner (22), and β-carotene (500 μM) was able to increase ^{3}H-DG uptake by 17% (Fig. 2C). The time-course of ^{3}H-FA accumulation by MDA-MB-231 cells was first determined. For this, cells were incubated with 20 nM ^{3}H-FA for various periods of time at two different pH: 7.5 and 5.5. ^{3}H-FA uptake was found to be time-dependent and pH-independent (Fig. 2D). A linear ^{3}H-FA uptake was observed in the first 6 min of incubation at both pH. With this information, we selected 6 min incubation time as the standard time and pH=7.5 to evaluate ^{3}H-FA uptake, and verified that β-carotene did not affect it (Fig.2E). **[Figure 2 near here]**

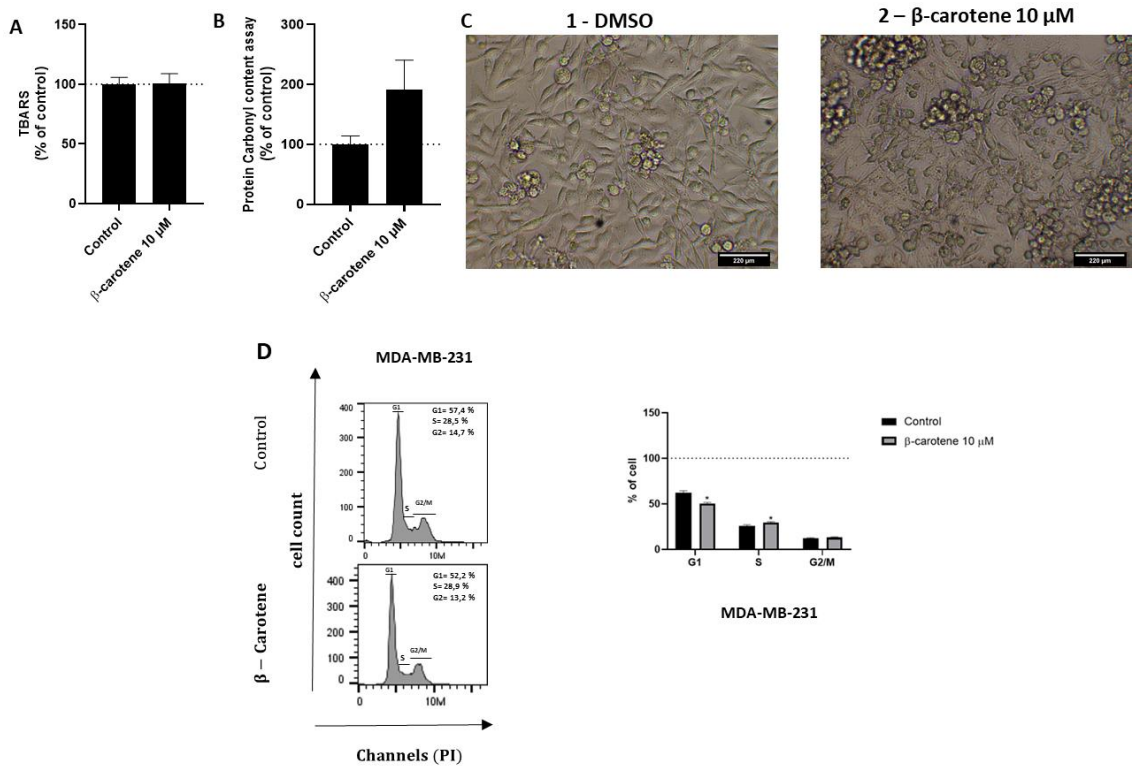


Further characterization of the antitumoral effect of β-carotene was made by testing only 10 µM of this compound.

Effect of β-carotene on MDA-MB-231 cells oxidative stress levels, cell morphology and cell cycle distribution

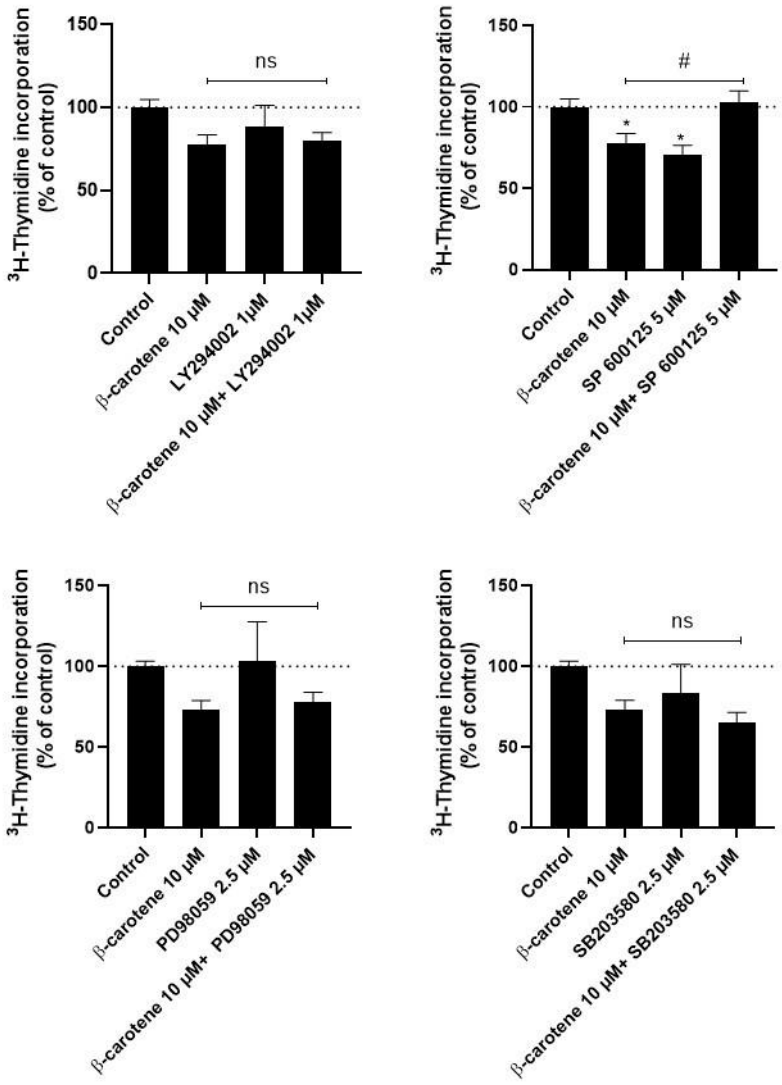
β-carotene (10 µM) had no effect significant effect on oxidative stress levels, evaluated by quantification of lipid peroxidation (TBARS) (Fig. 3A) and protein carbonyl levels (Fig. 3B). On the other hand, β-carotene (10 µM) slightly modified the morphology of MDA-MB-231 cells. Indeed, an irregular shape of β-carotene treated cells (Fig. 3C-2), compared to normal spindle-shaped morphology in 2D cultures (Fig. 3C-1), was observed (23). Finally, the cell cycle distribution of control MDA-MB-231 cells was 62 % in G1 phase, 26 % in S phase and 12 % in G2/M phase, and the cell cycle distribution of β-carotene-treated MDA-MB-231 cells was 50 % in G1 phase, 30 % in S phase and 13 % in G2/M phase (Fig. 3D). So, the cell cycle was affected by β-carotene (10 µM), through an increase in the amount of cells retained in S phase and a decrease of cells in G1 phase.

These results indicate that β-carotene induces cell cycle arrest at S phase. **[Figure 3 near here]**



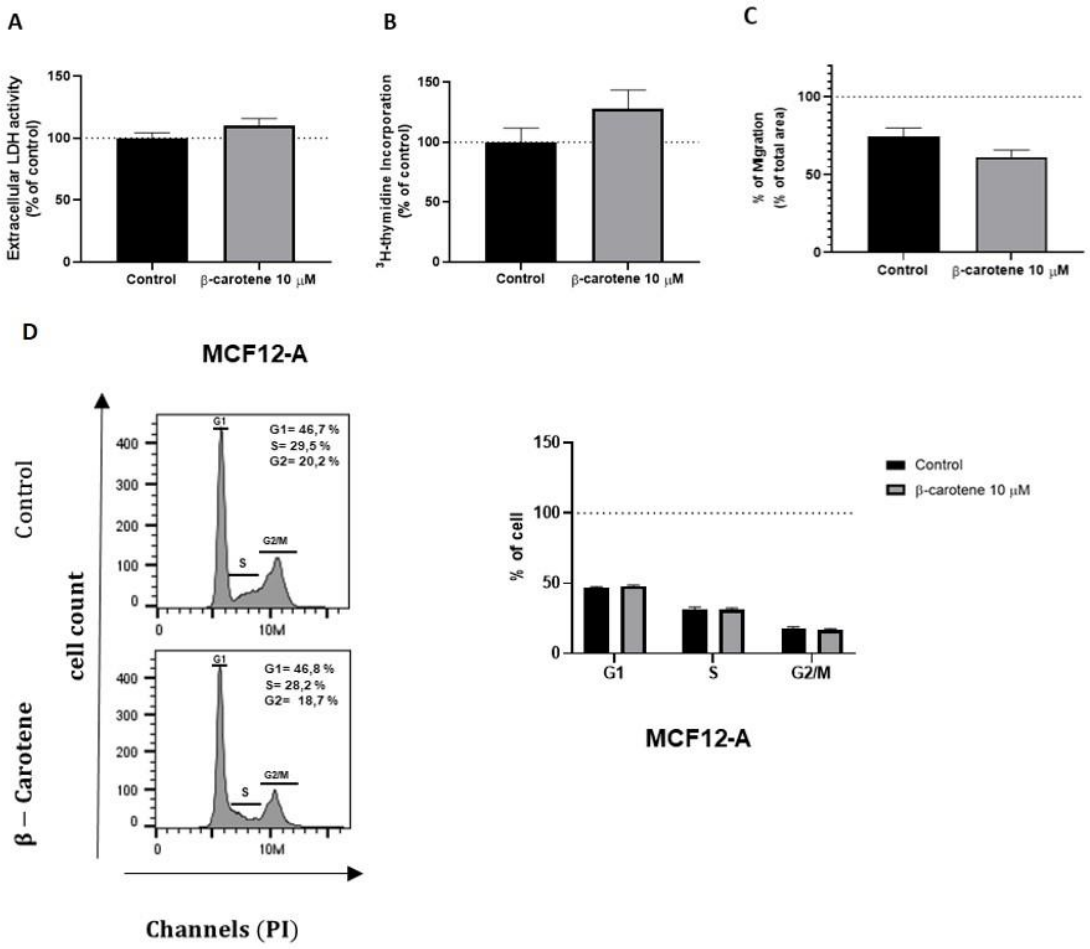
Determination of the putative involvement of the intracellular signalling pathways PI3K, ERK/MEK1/MEK2, p38 MAPK and JNK on the antiproliferative effect of β-carotene in MDA-MB-231 cells

The possible involvement of the intracellular signalling pathways PI3K, ERK/MEK1/MEK2, p38 MAPK and JNK in the antiproliferative effect of β-carotene was next investigated, by using specific inhibitors of these pathways (LY294002, PD98059, SB203580 and SP 600125, respectively). The antiproliferative effect of β-carotene was abolished in the presence of SP 600125 (5 µM), but was not affected by the other inhibitors (Fig. 4). These results thus suggest that the antiproliferative effect of β-carotene involves activation of the JNK pathway, but not of the other pathways. **[Figure 4 near here]**



Effect of β-carotene on MCF-12A cells

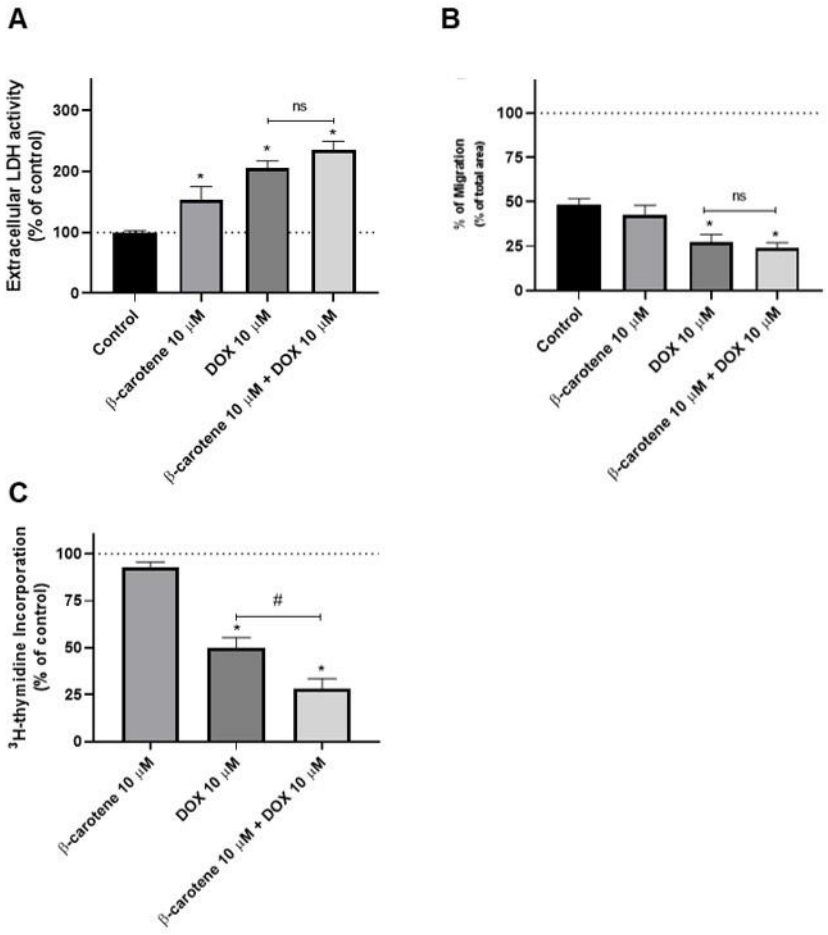
In this series of experiments, the effect of β-carotene (10 µM) on cell viability, proliferation, migration rates and cell cycle of the MCF-12A non-tumoral cell line was investigated. As shown in Figure 5, cell viability (Fig. 5A), proliferation (Fig. 5B), migration rates (Fig. 5C) and cell cycle (Fig. 5D) were not affected by β-carotene (10 µM) in these cells. **[Figure 5 near here]**



Effect of β-carotene in combination with doxorubicin on MDA-MB-231 cell proliferation

In this final series of experiments, the combined effect of β-carotene (10 μM) and doxorubicin (10 μM), the most commonly chemotherapeutic drug used on breast cancer treatment, on MDA-MB-231 viability, proliferation and migration was investigated. In relation to cell viability (Fig. 6A) and migration (Fig. 6B), the effect of doxorubicin was not statistically different when combined with β-carotene. In contrast, combination of β-carotene with doxorubicin enhanced the anti-proliferative effect of doxorubicin (Fig. 6C). These data suggest an additive anti-proliferative effect of β-carotene and doxorubicin.

[Figure 6 near here]



Discussion

Breast cancer is a heterogeneous disease. The insensitivity of TNBC to antihormonal treatment is an obstacle to treatment of this cancer, and consequently a high mortality rate is still observed for this breast cancer subtype. An alternative being investigated is the potential use of isolated dietary compounds on cancer treatment. Carotenoids have a significant antitumoral effect mediated by a wide range of molecular mechanisms modulating oxidative stress and redox balance, cellular signaling proteins, transcription factors, apoptosis, cell cycle progression and proliferation, angiogenesis, metastasis, gap junction intercellular communication and multidrug resistance (24). Based on these previous data, we selected the carotenoids β-carotene, crocin, astaxanthin and fucoxanthin to evaluate their effect on cell growth, viability, proliferation and migration of the MDA-MB-231 cell line.

With reference to culture growth, β-carotene (10-100-500 μM) and crocin (1-100 μM) decreased it. These results are in agreement with previous studies (24, 25). Also, β-carotene (500 μM) decreased cell viability, confirming a previous study on this cell line (24). When evaluating cell proliferation, β-carotene decreased cell proliferation (1-10-100-500 μM) but no concentration-dependency was observed. These results are in agreement with data from another work, where β-carotene has an antiproliferative effect even at a lower concentration (1 μM) (18). Fucoxanthin (1-10 μM) increased cell proliferation. A different effect was described by Déléris et al., who observed that fucoxanthin reduced the proliferation of the B16F10 melanoma cell line by inducing cell cycle arrest at the G0/G1 phase (26). In relation to cell migration, β-carotene (500 μM) and crocin (10 – 500 μM) decreased this parameter, which agrees with previous studies on other cell lines, namely SK-N-BE (2)C neuroblastoma (27) and GS and HGC-27 gastric cancer cell lines (28). Based on the results obtained, we selected β-carotene for further studies, because it decreases the viability and inhibits growth, proliferation and migration of MDA-MB-231 cells, thus revealing an antitumor effect.

As cancer cells have high glutamine, glucose and folate requirements, the effect of β-carotene in ^{3}H-GLN, ^{3}H-DG and ^{3}H-FA uptake was next assessed.

The main glucose transporter GLUT1 is highly expressed in MDA-MB-231 breast cancer cells (29, 30). We verified that β-carotene (500 μM) increased ^{3}H-DG uptake by 17%. This apparently contradictory increase in ^{3}H-DG uptake associated with a decrease in the amount of viable cells is similar to what happens in small cell lung cancer cells, where combinations of insulin with either metformin, pioglitazone or dichloroacetate resulted in an increase in glucose uptake combined with increased apoptosis (31). It is possible that the increase in cellular glucose uptake is a mechanism aiming at counteracting cell death.

The main transporter for glutamine in cancer cells is ASCT2, a Na^+-coupled transporter and a target of the oncogene c-Myc (6, 32). LAT1 is a Na^+ -independent glutamine transporter also used by cancer (33). In this study, the experiments were performed in both the presence and absence of Na^+, to evaluate the effect of β-carotene on both Na^+-dependent and Na^+-independent transporters. In the presence of Na^+, no significantly differences were observed on ^{3}H-GLN uptake. In the absence of Na^+, a decrease of ^{3}H-GLN uptake was observed with the lowest concentration of β-carotene (1 μM). However, it is important to mention that this effect is most probably not relevant, because ^{3}H-GLN uptake is mainly Na^+-dependent.

The time-course of ^{3}H-FA accumulation by MDA-MB-231 cells revealed that its uptake is time-dependent, but no pH-dependency was observed. We verified that β-carotene did not have a significant effect on ^{3}H-FA uptake at physiological pH. On a previous work using MCF-7 cells, a luminal breast cancer cell line, ^{3}H-FA was accumulated in a time-dependent manner at pH=7.5, whereas at pH 5.5 ^{3}H-FA uptake was almost undetectable and time-independent (34).

Carotenoids can decrease the oncogenic progress through the modulation of oxidative stress, cell cycle progression, apoptosis, angiogenesis, and metastasis (17). Based on this, the next step of this work was to better characterize the antitumoral effect of β-carotene (10 μM), by evaluating its effect on oxidative stress levels, cell morphology and cell cycle distribution, by determining which intracellular signalling pathways are involved on its antitumoral effects and by determining its effect when combined with doxorubicin. Moreover, the effect of β-carotene (10 μM) was also tested on a non-tumoral human breast epithelial cell line (MCF12-A cells), regarding cell viability, proliferation, migration and cell cycle.

Oxidative stress levels are known to interfere with the initiation, development and progression of cancer (35). More specifically, when in excess, ROS induce cellular damage and modifications of gene expression that affect DNA repair and cell proliferation, resulting on an enhanced risk of cancer initiation (35, 36). We verified that β-carotene did not change oxidative stress levels, as assessed by lipid peroxidation levels and carbonyl protein content assay, revealing that the antitumoral effect of this compound is not correlated with its antioxidant effect. The lack of antioxidant effect of β-carotene was not expected because of the previously demonstrated antioxidant properties of this compound, at normal oxygen pressure conditions (37, 38). However, when submitted to high oxygen pressures or at high concentrations, this compound induces loss of antioxidant properties and develops a prooxidant effect (37, 39).

In relation to cell morphology, β-carotene slightly modified this characteristic of MDA-MB-231 cells, which presented an altered cell shape compared to usually spindle-shaped form of non-treated cells (23). The interference of β-carotene with cell morphology was described previously in the cardiomyoblast cell line (H9C2) (40) and in the NCI-H69 small cell lung cancer cells (more vacuoles and lower nucleus: cytoplasm ratio, which correlated with a lower proliferation rate) (41).

The effect of β-carotene on cell cycle was also investigated. Interestingly, β-carotene was found to induce an increase of cells (≈ 3,85%) retained in S phase, followed by a decrease of cells (≈12,04%) occupying the G1 phase. These results support our previous results, showing an antiproliferative effect of β-carotene in MDA-BM-231 cancer cells. In a previous study, β-carotene was also described as having the capacity to inhibit MDA-MB-231 cell proliferation and to promote cell cycle arrest, through an increase of cells retained in the G2/M phase, followed by a decrease of cells in G0/G1 phase (42). From previous results on literature, cell cycle appears to be modulated by the

action of β-carotene, through multiple mechanisms (42). These mechanisms are already described for some cancer cell lines, including COLO 320 HSR (derived from human colon adenocarcinoma), where a G2/M phase arrest occurs, through downregulation of expression of cyclin A, AtT-20 (derived from a mouse anterior pituitary tumor), where S and G2/M phase arrest occurs, trough downregulation of expression of Skp2, and HL-90 (derived from human promyelocytic leukemia), where β-carotene caused G1 phase arrest (43).

Carotenoids can interfere with cancer intracellular signalling pathways. So, the putative involvement of signaling pathways known to be involved on antitumoral effects of β-carotene, namely, PI3Ks, JNK, ERK/MEK1/MEK2 and p38 MAPK pathways was investigated, by testing the effect of specific inhibitors of these pathways (LY294002, SP600125, PD98059, SB203580, respectively). The results obtained allow us to conclude that activation of the JNK pathway is involved in the antiproliferative effect of β-carotene in MDA-MB-231 cells. The JNK pathway, a subfamily of mitogen-activated protein kinases (MAPK), interferes with several aspects of cancer progression (namely, apoptosis, survival, proliferation, invasion and migration) in several types of cancer cells, including lung cancer, skin cancer and breast cancer (44). Specifically on TNBC cells, the JNK pathway appears to be involved in the invasiveness of this subtype of cancer. JNK can thus be a potential target for anticancer therapy (44). One commonly used JNK inhibitor, SP600125, is an ATP-competitive inhibitor that inhibits the phosphorylation of all JNK substrates with demonstrated effects on several cancers, including stomach and pancreatic cancer and glioblastoma (44). High oxidative stress levels, associated with elevated ROS production, were previously correlated with the activation of JNK pathway (35). However, our data suggest that another mechanism is involved in β-carotene-

induced activation of JNK, because β-carotene did not increase oxidative stress levels but caused activation of JNK pathway.

A comparative study with a non-tumorigenic cell line (MCF-12A) was also performed, in order to determine if the effects of β-carotene are cancer cell-selective. When tested on this cell line, β-carotene (10 μM) did not affect cell viability, proliferation and migration rates and also did not interfere with the cell cycle. These data (together with previous data with cultured hepatocytes (45)) thus suggest that β-carotene does not have a negative effect on non-tumorigenic cells and selectively kills cancer cells.

Due to low therapeutic options for TNBC, there is a huge necessity in finding compounds with anticancer activity against this breast cancer subtype (46). One of these compounds is doxorubicin, an anthracycline drug commonly used on chemotherapy regimens for breast cancer (46, 47). However, this drug presents limited applications due to its toxicity on non-cancer cells and serious side effects on patients, including cardiotoxicity (48). So, other compounds, which permit the selective killing of cancer cells and the reduction of side effects, need to be found. Carotenoids, used alone or in combination with doxorubicin, could eventually have this desired effect (46). In our study, we evaluated cell viability, proliferation and migration rates, when MDA-MB-231 cells were submitted to a combination treatment of β-carotene with doxorubicin (10 μM). In relation to cell viability and migration, β-carotene (10 μM), when conjugated with doxorubicin, did not enhance the cytotoxic and the antimigratory effect of doxorubicin. This is not in agreement with data on literature, where an exposure of MDA-MB-231 cells to β-carotene (20 μM) in combination with doxorubicin (10 μM) promoted a higher decrease of cell viability, when compared to doxorubicin and β-carotene alone (46). This difference could be related to the fact that a higher concentration of β-carotene was used in the previous report. Of importance, β-carotene (10 μM) in combination with

doxorubicin potentiated the anti-proliferative effect of doxorubicin, suggesting a possible additive anti-proliferative effect. So, these results suggest that β-carotene may have a potential role as a co-adjuvant to doxorubicin for TNBC therapy, because β-carotene has cancer-selective effects and shows an additive anti-proliferative effect with doxorubicin in relation to the MDA-MB-231 cell line.

We can conclude that β-carotene decreases cell viability, proliferation, culture growth and cell migration of MDA-MB-231 cells. β-carotene significantly increases ^{3}H-DG uptake and does not affect ^{3}H-GLN and ^{3}H-FA uptake. So, the anti-tumoral effect of β-carotene in relation to a TNBC cell line is not correlated with a decrease in the cellular uptake of important nutrients (glucose, glutamine and folic acid) for these cells. Moreover, it is also not correlated with an antioxidant effect, but is dependent on activation of the JNK intracellular pathway. Also, β-carotene affects cell cycle distribution of the MDA-MB-231 cell line. Importantly, β-carotene did not affect cell viability, proliferation, migration rates and cell cycle distribution of MCF12-A cells, revealing that the effect of β-carotene is cancer cell-selective. We also observed that β-carotene reveals a possible additive anti-proliferative effect in combination with doxorubicin. So β-carotene could be used as a co-adjuvant therapy with doxorubicin, increasing its anti-proliferative effect without affecting non-cancerous cells.

References

[1] Diana A, Franzese E, Centonze S, Carlino F, Della Corte CM, Ventriglia J, et al. Triple-negative breast cancers: systematic review of the literature on molecular and clinical features with a focus on treatment with innovative drugs. *Curr Oncol Rep*. 2018;20(10):76.

[2] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021.

[3] Senkus E, Kyriakides S, Penault-Llorca F, Poortmans P, Thompson A, Zackrisson S, et al. Primary breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2013;24 Suppl 6:vi7-23.

[4] Barbosa AM, Martel F. Targeting glucose transporters for breast cancer therapy: the effect of natural and synthetic compounds. *Cancers (Basel)*. 2020;12(1).

[5] Cazzaniga M, Bonanni B. Breast cancer metabolism and mitochondrial activity: the possibility of chemoprevention with metformin. *Biomed Res Int*. 2015;2015:972193.

[6] Cha YJ, Kim ES, Koo JS. Amino acid transporters and glutamine metabolism in breast cancer. *Int J Mol Sci*. 2018;19(3).

[7] Still ER, Yuneva MO. Hopefully devoted to Q: targeting glutamine addiction in cancer. *Br J Cancer*. 2017;116(11):1375-81.

[8] Scalise M, Pochini L, Galluccio M, Console L, Indiveri C. Glutamine transport and mitochondrial metabolism in cancer cell growth. *Frontiers in Oncology*. 2017;7.

[9] Liu Y, Zhao T, Li Z, Wang L, Yuan S, Sun L. The role of ASCT2 in cancer: a review. *Eur J Pharmacol*. 2018;837:81-7.

[10] Augustin R. The protein family of glucose transport facilitators: It's not only about glucose after all. *IUBMB Life*. 2010;62(5):315-33.

[11] Carvalho KC, Cunha IW, Rocha RM, Ayala FR, Cajaíba MM, Begnami MD, et al. GLUT1 expression in malignant tumors and its use as an immunodiagnostic marker. *Clinics (Sao Paulo)*. 2011;66(6):965-72.

[12] Pragallapati S, Manyam R. Glucose transporter 1 in health and disease. *J Oral Maxillofac Pathol*. 2019;23(3):443-9.

[13] Tomita LY. Folate and cancer. *J Inborn Errors Metab Screen*. 2016;4.

[14] Rosenzweig A, Blenis J, Gomes AP. Beyond the Warburg Effect: How do cancer cells regulate one-carbon metabolism? *Front Cell Dev Biol*. 2018;6.

[15] Koseki J, Konno M, Asai A, Colvin H, Kawamoto K, Nishida N, et al. Enzymes of the one-carbon folate metabolism as anticancer targets predicted by survival rate analysis. *Sci Rep*. 2018;8(1):303.

[16] Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. Drug Resistance Updates. 2012;15(4):183-210.

[17] Saini RK, Keum YS, Daglia M, Rengasamy KR. Dietary carotenoids in cancer chemoprevention and chemotherapy: a review of emerging evidence. *Pharmacol Res*. 2020;157:104830.

[18] Sowmya Shree G, Yogendra Prasad K, Arpitha HS, Deepika UR, Nawneet Kumar K, Mondal P, et al. β-carotene at physiologically attainable concentration induces apoptosis and down-regulates cell survival and antioxidant markers in human breast cancer (MCF-7) cells. *Mol Cell Biochem*. 2017;436(1-2):1-12.

[19] Nogueira J, Soares SF, Amorim CO, Amaral JS, Silva C, Martel F, et al. Magnetic driven nanocarriers for ph-responsive doxorubicin release in cancer therapy. *Molecules*. 2020;25(2):333.

[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal*. 1976;72(1):248-54.

[21] Silva C, Andrade N, Rodrigues I, Ferreira AC, Soares ML, Martel F. The pro-proliferative effect of interferon-γ in breast cancer cell lines is dependent on stimulation of ASCT2-mediated glutamine cellular uptake. *Life Sci*. 2021;286:120054.

[22] Silva C, Andrade N, Guimarães JT, Patrício E, Martel F. The in vitro effect of the diabetes-associated markers insulin, leptin and oxidative stress on cellular characteristics promoting breast cancer progression is GLUT1-dependent. *Eur J Pharmacol*. 2021;898:173980.

[23] Ivers LP, Cummings B, Owolabi F, Welzel K, Klinger R, Saitoh S, et al. Dynamic and influential interaction of cancer cells with normal epithelial cells in 3D culture. *Cancer Cell Int*. 2014;14(1):108.

[24] Maghsoudi S, Taghavi Shahraki B, Rabiee N, Fatahi Y, Bagherzadeh M, Dinarvand R, et al. The colorful world of carotenoids: a profound insight on therapeutics and recent trends in nano delivery systems. *Crit Rev Food Sci Nutr*. 2021:1-40.

[25] Hoshyar R, Mollaei H. A comprehensive review on anticancer mechanisms of the main carotenoid of saffron, crocin. *J Pharm Pharmacol*. 2017;69(11):1419-27.

[26] Déléris P, Nazih H, Bard JM. Chapter 10 - Seaweeds in human health. In: Fleurence J, Levine I, editors. *Seaweed in Health and Disease Prevention*. San Diego: Academic Press; 2016. p. 319-67.

[27] Kim Y-S, Lee H-A, Kim Y. The effect of beta-carotene on migration and invasion in SK-N-BE(2)C neuroblastoma cells. *The FASEB Journal*. 2013;27(S1):639.16-.16.

[28] Zhou Y, Xu Q, Shang J, Lu L, Chen G. Crocin inhibits the migration, invasion, and epithelial-mesenchymal transition of gastric cancer cells via miR-320/KLF5/HIF-1α signaling. *J Cell Physiol*. 2019;234(10):17876-85.

[29] Macheda ML, Rogers S, Best JD. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol*. 2005;202(3):654-62.

[30] Furuta E, Okuda H, Kobayashi A, Watabe K. Metabolic genes in cancer: their roles in tumor progression and clinical implications. *Biochim Biophys Acta*. 2010;1805(2):141-52.

[31] Domvri K, Zogas N, Zarogoulidis P, Petanidis S, Porpodis K, Kioseoglou E, et al. AB060. Could glucose uptake ability enhanced by drugs result in antitumor effect in small cell lung cancer? *Ann Transl Med*. 2016;4(22).

[32] Dong Y, Tu R, Liu H, Qing G. Regulation of cancer cell metabolism: oncogenic MYC in the driver's seat. *Signal Transduct Target Ther*. 2020;5(1):124.

[33] Häfliger P, Charles R-P. The L-Type Amino Acid Transporter LAT1-An Emerging Target in Cancer. *Int J Mol Sci*. 2019;20(10):2428.

[34] Guedes M, Araujo JR, Correia-Branco A, Gregorio I, Martel F, Keating E. Modulation of the uptake of critical nutrients by breast cancer cells by lactate: Impact on cell survival, proliferation and migration. *Exp Cell Res*. 2016;341(2):111-22.

[35] Arfin S, Jha NK, Jha SK, Kesari KK, Ruokolainen J, Roychoudhury S, et al. Oxidative stress in cancer cell metabolism. *Antioxidants*. 2021;10(5):642.

[36] Klaunig JE. Oxidative stress and cancer. *Curr Pharm Des*. 2018;24(40):4771-8.

[37] Burton GW, Ingold KU. β-Carotene: an unusual type of lipid antioxidant. *Science*. 1984;224(4649):569-73.

[38] Erhardt JG, Mack H, Sobeck U, Biesalski HK. β-Carotene and α-tocopherol concentration and antioxidant status in buccal mucosal cells and plasma after oral supplementation. *Br J Nutr*. 2002;87(5):471-5.

[39] Palozza P, Serini S, Di Nicuolo F, Piccioni E, Calviello G. Prooxidant effects of β-carotene in cultured cells. *Mol Aspects Med*. 2003;24(6):353-62.

[40] Lesmana R, Yusuf I, Goenawan H, Achadiyani A, Khairani AF, Fatimah S, et al. Low dose of β-carotene regulates inflammation, reduces caspase signaling, and correlates with autophagy activation in cardiomyoblast cell lines. *Med Sci Monit*. 2020;26.

[41] Prakash P, Manfredi TG, Jackson CL, Gerber LE. β-carotene alters the morphology of NCI-H69 small cell lung cancer cells. *J Nutr*. 2002;132(1):121-4.

[42] Gloria NF, Soares N, Brand C, Oliveira FL, Borojevic R, Teodoro AJ. Lycopene and beta-carotene induce cell-cycle arrest and apoptosis in human breast cancer cell lines. *Anticancer Res*. 2014;34(3):1377-86.

[43] Niranjana R, Gayathri R, Nimish Mol S, Sugawara T, Hirata T, Miyashita K, et al. Carotenoids modulate the hallmarks of cancer cells. *J Funct*. 2015;18:968-85.

[44] Wu Q, Wu W, Jacevic V, Franca TCC, Wang X, Kuca K. Selective inhibitors for JNK signalling: a potential targeted therapy in cancer. *J Enzyme Inhib Med Chem*. 2020;35(1):574-83.

[45] Yang S-C, Huang C-C, Chu J-S, Chen J-R. Effects of β-carotene on cell viability and antioxidant status of hepatocytes from chronically ethanol-fed rats. *B J Nutr*. 2004;92(2):209-15.

[46] Vijay K, Sowmya PR-R, Arathi BP, Shilpa S, Shwetha HJ, Raju M, et al. Low-dose doxorubicin with carotenoids selectively alters redox status and upregulates oxidative stress-mediated apoptosis in breast cancer cells. *Food Chem Toxicol*. 2018;118:675-90.

[47] Liu M, Yin H, Qian X, Dong J, Qian Z, Miao J. Xanthohumol, a prenylated chalcone from hops, inhibits the viability and stemness of doxorubicin-resistant MCF-7/ADR Cells. *Molecules*. 2016;22(1).

[48] Alkaraki A, Alshaer W, Wehaibi S, Gharaibeh L, Abuarqoub D, Alqudah DA, et al. Enhancing chemosensitivity of wild-type and drug-resistant MDA-MB-231 triple-negative breast cancer cell line to doxorubicin by silencing of STAT 3, Notch-1, and β-catenin genes. *J Breast Cancer*. 2020;27(5):989-98.

Figure Legends

Figure 1 - Effect of carotenoids on culture growth, viability, proliferation and migration of MDA-MB-231 cells. (A) Effect of exposure to carotenoids on culture growth. After a 24h exposure to carotenoids (1-10-100-500 µM) or solvent, MDA-MB-231 culture growth was determined by cellular protein quantification using the sulforhodamine B (SRB) assay (n=9-12). Results are present presented as absorbance (% of control). **(B) Effect of carotenoids on cell viability.** After a 24h exposure to carotenoids (1-10-100-500 µM) or solvent, MDA-MB-231 cell viability was determined by quantification of extracellular LDH activity (n=9-12). Results are presented as LDH activity (% of control). **(C) Effect of carotenoids on cell proliferation rates.** After 24h exposure to carotenoids (1-10-100-500 µM) or solvent, MDA-MB-231 cell proliferation rate was determined by quantification of ^{3}H-thymidine incorporation (n=9-12). Results are presented as µCi/ mg protein (% of control). **(D) Effect of carotenoids on cell migration.** A scratch in cell culture was made at 0 h, and cells were then exposed to the carotenoids (1, 10, 100 or 500 µM), or to the solvent, for 24h. Pictures were taken at 0h and 24h (n=6-9). Results are presented as % of migration (% of total area). **(E) Representative images of cell migration.** A scratch in cell culture was made at 0 h, and cells were then exposed to the carotenoids (1, 10, 100 or 500 µM), or to the solvent, for 24h. Pictures were taken at 0h and 24h. Shown are the representative images obtained with each carotenoid (500 µM) (magnification 10 X). Shown are arithmetic means ±SEM. * Significantly different from control ($P<0.05$).

Figure 2 - Effect of β-carotene on ^{3}H-GLN, ^{3}H-DG and ^{3}H-FA uptake of MDA-MB-231 cells. (A) ^{3}H-GLN cellular uptake, determined in the presence (Total) and absence of Na^{+} (Na^{+}-independent). Na^{+}-dependent uptake was calculated by subtracting Na^{+}-independent from total uptake. Results are presented as pmol/mg prot. Shown are arithmetic means ±SEM (n=4). **(B) Effect of β-carotene on ^{3}H-GLN cellular uptake.** After a 24h exposure to β-carotene (1-10-100-500 µM) or solvent, cells were preincubated for 20 min followed by an incubation for 6 min with 5 nM ^{3}H-GLN (with or without Na^{+}), also in the presence of β-carotene or solvent (n=6).

Results are presented as pmol/mg protein (% of control). **(C) Effect of β-carotene on ^{3}H-DG cellular uptake.** After a 24h exposure to β-carotene (1-10-100-500 µM) or solvent, cells were preincubated for 20 min followed by an incubation for 6 min with 10 nM ^{3}H-DG (n=16), also in the presence of β-carotene or solvent. Results are presented as pmol/mg protein (% of control). **(D) Time-course of ^{3}H-FA uptake by MDA-MB-231 cells.** Cells were incubated at 37°C with 20 nM ^{3}H-FA for 2, 5, 10, 15, 20 and 60 min, at two different extracellular pH (pH=5.5/pH=7.5). Shown are arithmetic means ± SEM (n=8). **(E) Effect of β-carotene on ^{3}H-FA cellular uptake.** After a 24h exposure to β-carotene (1-10-100-500 µM) or solvent, cells were preincubated for 20 min followed by an incubation for 6 min with 20 nM ^{3}H-FA (n=12), also in the presence of β-carotene or solvent. Results are presented as pmol/mg protein (% of control). Shown are arithmetic means ±SEM. * Significantly different from control ($P<0.05$).

Figure 3 - Effect of β-carotene on oxidative stress levels, cell morphology and cell cycle distribution of MDA-MB-231 cells. (A) Effect of β-carotene on oxidative stress levels, assessed with the TBARS assay. After a 24h exposure to β-carotene (10 µM) or solvent, lipid peroxidation levels were determined (n=9). **(B) Effect of β-carotene on oxidative stress levels, assessed with the protein carbonyl content assay.** After a 24h exposure to β-carotene (10 µM) or solvent, protein carbonyl levels were determined (n=12). Shown are arithmetic means ±SEM. * Significantly different from control (DMSO) ($P<0.05$). **(C) Effect of β-carotene on morphology of MDA-MB-231 cells.** After a 24 h exposure to β-carotene (2) (10 µM) or solvent (1), the cell culture was visualized (magnification 20 X). **(D) Effect of β-carotene on MDA-MB-231 cell cycle distribution (n=5-6).** After a 24 h exposure to β-carotene (10 µM) or solvent, the cell cycle distribution was assessed by flow cytometry. Shown are arithmetic means ± SEM.* Significantly different from control ($P < 0.05$).

Figure 4 - Effect of inhibitors of the intracellular signalling pathways PI3K (LY294002), ERK/MEK1/MEK2 (PD98059), p38 MAPK (SB203580) and JNK (SP 600125) on the antiproliferative effect of β-carotene. After a 24h exposure to β-carotene (10 µM) (or solvent)

and/or inhibitors, cell proliferation rates were determined (n=9). Results are presented as µCi/ mg protein (% of control). Shown are arithmetic means ±SEM. * Significantly different from control ($P<0.05$); # Significantly different from each other; ns not different

Figure 5 - Effect of β-carotene on viability, proliferation, migration rates and cell cycle distribution of MCF-12A cells. (A) Effect of β-carotene on cell viability (n=8-12). Results are presented as LDH activity (% of control)**. (B) Effect of β-carotene on cell proliferation rates.** Results are presented as ^{3}H-thymidine incorporation (µCi/mg protein) (% of control). (n=9). **(C) Effect of β-carotene on cell migration rates.** Results are presented as % of total area (n=9). **(D) Effect of β-carotene on cell cycle distribution** (n=5-6). Shown are arithmetic means ± SEM. *Significantly different from control ($P < 0.05$).

Figure 6 - Effect of β-carotene and/or doxorubicin on MDA-MB-231 cell viability, proliferation and migration rates. (A) Effect of β-carotene and/or doxorubicin (DOX) on cell viability. After a 24h exposure to β-carotene (10 µM) and/or DOX (10 µM), cell viability was determined by quantification of extracellular LDH activity (n=9). Results are presented as LDH activity (% of control). **(B) Effect of β-carotene and/or doxorubicin (DOX) on cell migration rates.** A scratch in cell culture was made at 0 h, and cells were then exposed to the compounds, or to the solvents, for 24h. Pictures were taken at 0h and 24h. Results are presented as % of total area (n=9). **(C) Effect of β-carotene and/or doxorubicin (DOX) on proliferation rates.** After 24h exposure to compounds or solvents, cell proliferation rates were determined by quantification of ^{3}H-thymidine incorporation. Results are presented as ^{3}H-thymidine incorporation (µCi/mg protein) (% of control). (n=9). Shown are arithmetic means ± SEM. *Significantly different from control ($P < 0.05$).