Original Manuscript Link:
https://pubs.rsc.org/en/content/articlelanding/2018/fo/c7fo01804e#!divAbstract

DOI: https://doi.org/10.1039/C7FO01804E

PRE-PRINT MANUSCRIPT

Natural and synthetic avenanthramides activate caspases 2, 8, 3 and downregulate hTERT, MDR1 and COX-2 genes in CaCo-2 and Hep3B cancer cells

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Abstract

Avenanthramides (AVNs) are amino phenolic compounds, which can be isolated from oat sprouts in a mixture, mainly represented by the AVN 2p, 2c and 2f. The three forms can also be chemically synthesized (2p-S; 2c-S; 2f-S). In the present study, we compared the antioxidant, antiproliferative and anti-inflammatory activities of the synthesized and the natural AVN mixture. The AVN 2c-S had the highest Oxygen Radical Absorbance Capacity (ORAC), whereas the intracellular antioxidant activity (DCFH-DA assay) provided similar values for all AVNs. The AVN 2c-S had the highest ORAC value, whereas the DCFH-DA assay provided similar values for all AVNs. The anticancer effectiveness of AVNs was investigated on CaCo-2 and Hep3B cancer cell lines. Hep3B cells were more sensitive than CaCo-2 cells to the antiproliferative effect of AVNs. In Hep3B cells no significant difference was observed among the synthetized and natural AVNs, whereas some differences were observed in CaCo-2 cancer cell line, with AVN 2c-S showing the greatest antiproliferatiuve effect. In both CaCo-2 and Hep3B cells, natural and synthetic AVNs activated caspases 8 and 3, with AVN mix and AVN 2c-S able to activate also caspase 2. Results indicated that both synthetic and natural AVNs reduced expression levels of the pro-survival genes hTERT, COX-2 and MDR1, as well as inhibited the activity of the pro-inflammatory COX-2 enzyme, with slight differences. The AVNs are chemopreventive nutrients, able to provide a pleiotropic

anticancer effect, which is worthy to be investigated in *in vivo* animal models of colon adenocarcinoma and hepatocarcinoma.

Keywords: avenanthramides, antioxidant, anticancer, anti-inflammatory, CaCo-2, Hep3B, apoptosis

Introduction

Oat (*Avena sativa L.*) is an important source of both soluble and insoluble dietary fibers, as well as of several other bioactive constituents, namely tocols, flavonoids, phenolic acids and avenanthramides (AVNs) [Peterson, D.M., 2001]. AVNs contain an anthranilic acid and a cinnamic acid, but the substitutions on the two molecules distinguish them in 25 different AVN compounds. The most abundant AVNs in oats are: N-(3',4'-dihydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (2c; also indicated as C), N-(4'-hydroxy-3'-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (2f; also indicated as B) and N-(4'-hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (2p; also indicated as A) [Collins, F.W., 1989]. The bioavailability of AVNs has been demonstrated in hamsters, rats and humans [Chen CY, 2004; Chen CYO, 2007; Koenig RT, 2011]. AVNs exhibit several beneficial effects, including antioxidant, anti-inflammatory and antiproliferative effects [Meydani M., 2009; Guo, W. et al., 2010; Scarpa, ES et al., 2017].

In vitro antioxidant activity of AVNs has been extensively investigated through the DPPH assay [Chu et al., 2013; Dimberg et al., 1993; Peterson et al., 2002] and through the ORAC assay [Scarpa ES et al., 2017, Yang 2014, Food Chem].

In a previous work [Scarpa ES et al., 2017], we showed that the AVN natural mix was able to activate the extrinsic apoptotic pathway, which is regulated by the initiator caspase 8 and the effector caspase 3 [Samali A. et al., 2014]. Indeed, we observed the downregulation of the pro-survival markers HIF1A and VEGFA, but other anti-apoptotic and survival pathways could be modulated after phytochemical treatments. One of these survival mechanisms is associated with

high levels of telomerase expression [Hahn, W.C. et al., 1999]. In normal somatic cells, the telomeric DNA progressively shortens with each cell division, leading to cell death [Hahn, W.C. et al., 1999], whereas in the cancer cells telomeres are stable, through high levels of telomerase activity, thus providing immortalization and increasing the proliferation rate [Hahn, W.C. et al., 1999], as well as resistance against anticancer drugs [Kirkpatrick, K.L. et al., 2003]. Specific inhibitors of telomerase activity are actually used for cancer therapy [Hahn, W.C., Meyerson, M.; 2001].

Resistance to apoptosis is also linked to downreulation of the expression levels of the MDR1 gene, able to exploit the multidrug resistance (MDR) by effluxing anticancer agents outside the cells [Gottesman et al., 2002] through the P-glycoprotein (P-gp) [Dean et al., 2001], whereas downregulation of MDR1 expression levels can increase the efficacy of anticancer drugs [Wink et al., 2012].

The survival and over-proliferation of cancer cells could be also linked to the pro-inflammatory tumor microenvironment [Hanahan & Weinberg; 2011], mostly dependent on two cyclooxygenase (COX) isoforms, catalyzing the production of inflammatory prostaglandins from arachidonic acid. The constitutively expressed COX-1 maintains the homeostatic level of prostaglandins; whereas the inducible COX-2 is highly expressed in many types of solid cancers and contributes to tumor growth and invasiveness [Smith WL et al., 2000; Wang D et al., 2010], via inhibition of apoptosis and increase of angiogenesis [Zha, S. et al., 2014].

The aim of this work was to compare the cytotoxic activities of synthetized AVN forms 2c, 2f, 2p and natural AVN mixture, on CaCo-2 and Hep3B cancer cell lines. The former cells are characterized by a mutated TP53 gene and overexpression of anti-apoptotic factors [Piccirillo S. et al., 2009]; the latters produce an inactive p53 protein and show a high resistance to conventional anticancer drugs [Gambari R. et al., 2014]. The pro-apoptotic and anti-inflammatory activities of synthetic AVNs and natural AVN mix were deeply investigated through the measurement of the

caspase 2, 8, 3 activities and hTERT, MDR1 and COX-2 expression levels. The CaCo-2 cancer cells were already studied by us in the presence of the natural AVN mix only, while Hep3B were here investigated in the presence of AVNs for the first time. This study was undertaken in order to explore the possible use of AVNs in functional and nutraceutical products for chemoprevention.

Materials and methods

Reagents and chemicals

Ethanol and ethyl-acetate (analytical grade) were purchased from WWR International (Radnor, USA). DMEM culture medium, fetal bovine serum (FBS), antibiotics, trypsin, MEM non-essential amino acid solution, Sulforhodamine B sodium salt, water (LC-MS grade), acetonitrile (LC-MS grade), formic acid (LC-MS grade), glacial acetic acid, 2,2'-Azobis (2-amidinopropane) dihydrocloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid (Trolox), Fluorescein were purchased from Sigma-Aldrich (St. Louis, USA).

AVN purification

Oat sprouting

Oat grains, provided by the local producer Terra Bio Soc. Coop. (Urbino, Italy), were steeped and germinated for 14 and 72 hours, respectively, using an automatic sprouter (Fresh Life 2000, Corrupad Korea Co. Ltd., Korea), following the methods previously reported [9, 10]. Steeped and germinated oat grains were freeze-dried and stored at -20° C until analysis. Samples were milled in a ZM 200 ultracentrifugal mill with a 0.5 ring sieve (Retsch, Haan, Germany).

Extraction and isolation of AVNs

AVNs were extracted from milled oat with acidified ethanol (1:10 w/v), following the procedure reported by us [8], with the exception that ethanol was acidified with 0.1% glacial acetic acid, instead of 10 mM H_3PO_4 buffer.

The raw extract was concentrated by rotary evaporation to 1/10 of the initial volume. To remove lipophilic components from the extract, we followed the procedure reported by Liu et al [32], with some modifications, as previously described [Scarpa ES et al., 2017].

HPLC-PDA-MS analysis of AVNs

The dried fractions obtained from the Sephadex LH-20 column chromatography were dissolved in ethanol and directly analyzed in a Water instrument equipped with Alliance HT 2795 High Performance liquid Chromatography (HPLC), 2996 Photo Diode Array (PDA) and Micromass LC/MS ZQ 2000 detector, following the procedure reported by us [8].

To identify the individual AVNs in the HPLC chromatograms, retention time, UV spectra, MS ESI (+) and ESI (-) spectra were compared with those of commercially available external standards (AVN A, B, C, Sigma-Aldrich, St. Louis, USA), and the peak areas were used for quantification.

Syntheses of AVNs

Avenanthramides 2c, 2p, 2f **(Figure 1)** were synthesized according to a previous synthetic strategy (Wise et al 2011), with some modifications. Briefly, 2 ml of a 5 mM solution of the appropriate phenylpropanoid (p-coumaric, ferulic or caffeic acid) in pyridine were added to 10 ml of acetic anhydride and the mixture was stirred for 5 h at room temperature. Cold water (50 mL) was added and the solution was left at 5°C for one hour. The precipitate was collected by filtration and washed several times with cold water. The compound was dried overnight at 45°C under vacuum. The acetylated phenylpropanoid was then dissolved in 15 ml of dimethylformamide (DMF) with 2.1 mL of triethylamine (TEA). The solution was stirred at 0°C and an equimolar amount of (Benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyBOP) in solution in CH_2Cl_2 (36 mL) was added dropwise. Next, a solution of 5-hydroxyanthranilic acid (equimolar to the protected phenylpropanoid) in 15 mL of DMF was added dropwise to the solution. The solution was stirred at

0°C for 30 min, than for 2 h at room temperature. 0.5 M HCl was added (80 mL) and the mixture was stored overnight at 4°C. The acetoxy avenantramides were extracted with ethyl acetate and the solvent was removed under reduced pressure. Finally, the protecting acetyl groups were removed by dissolving the avenanthramides in MeOH: Water (1 : 1) 80 mL with the addition of 4 equivalents of Ammonium Acetate, and stirring over night at room temperature. The mixture was then acidified with 2N HCl and extracted with ethyl acetate. The solvent was removed under reduced pressure and the final compounds were purified by crystallization in MeOH and Water (**2p** and **2f**) or CH_2Cl_2 and Hexane (**2c**).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 Ultrashield spectrometer, using CD_3OD as solvent. Chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent. Coupling constants (*J* values) are given in hertz (Hz). ESI-MS spectra were recorded with a Waters Micromass ZQ instrument, molecular ions (M - 1) are given for ESI-MS analysis.

ORAC Assay

Antioxidant activity was assessed using the ORAC (Oxygen Radical Absorbance Capacity) assay and a Fluostar Optima plate reader fluorimeter (BMG Labtech, Offenburgh, Germany) equipped with a temperature-controlled incubation chamber and automatic injection pump according to Ninfali et al [33]. Fluorescence was read at 485 nm ex. and 520 nm em. until complete extinction. ORAC values were expressed as μmol Trolox Equivalents (TE) g^{-1} d.m, which was used as external standard.

Cell cultures

Hep3B liver cancer cell line and CaCo-2 colon cancer cell line were purchased from the American Type Culture Collection (ATCC, Rockville, USA). CaCo-2 were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and

100 μM MEM non-essential amino acid solution. Hep3B were maintained in the same medium, except for the addition of 200 μM MEM non-essential amino acid solution. Cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

Sulforhodamine B (SRB) assay

Cells (10^4/well for CaCo-2 and Hep3B) were plated in 96-well plates and treated with AVNs Mix, AVN 2c-S, AVN 2f-S, AVN 2p-S for 24, 48 and 72 hours. At the end of treatment, cells were incubated with SRB as previously reported [5]. The results were expressed as the percentage of cell viability, compared to control untreated cells (100% viability).

DCFH-DA Assay

CaCo-2 cells were seeded ($2x10^5$/well) in 6-well plates and treated for 24h with AVN mix or AVN 2c-S, or 2f-S, or 2p-S. After removing the cell medium, the H2O2-mediated production of ROS in CaCo-2 cells was monitored following the procedure previously reported [Scarpa ES et al., 2017]. The images were obtained using the software OLYMPUS Ps-blm1.

Activity detection of caspases 3, 8 and 2

CaCo-2 and Hep3B cells were untreated, or treated with AVNs Mix, AVN 2c-S, AVN 2f-S or AVN 2p-S for 24h and the cytosols were extracted as previously reported [Scarpa ES et al., 2017].

Caspase 3, 8 and 2 activities were assessed through the Caspase Colorimetric Assay Kit (BioVision, Milpitas, USA) according to the manufacturer's instructions. 100 μg of cytosol from untreated or AVN treated CaCo-2 and Hep3B cells were incubated with caspase reaction buffer (25 mM Hepes pH 7.4, 50 mM NaCl, 0.05% CHAPS, 0.5 mM EDTA, 5% glycerol, 5 mM DTT), then the substrate (200 μM final concentration) DMQD-pNA specific for caspase 3, or IETD-pNA specific for caspase 8, or VDVAD-pNA specific for caspase 2 was added. The samples were then transferred in a 96-wells microplate and incubated at 37ºC for 2h. The absorbance was measured at λ=405 nm in a microplate reader (BioRad Laboratories, Hercules, USA).

RNA extraction, cDNA production and RTqPCR assay

Total RNA was extracted from untreated or AVN treated CaCo-2 and Hep3B cells using TriReagent (Invitrogen, Carlsbad, USA), following the manufacturer's protocol. RNA was quantified and reverse transcribed following the previously reported method [Scarpa ES et al., 2017].

RTqPCR analysis of cDNA samples was performed using the KAPA SYBR Fast (2x) ABI PRISM qPCR Kit (KAPA Biosystems Inc., Cape Town, South Africa) and the ABI PRISM 7700 Sequence Detector (Applied Biosystems Inc., Foster City, USA). Thermal cycling conditions were as follows: 95ºC for 10', followed by 40 cycles of 15'' at 95ºC, 15'' at 60ºC, 15'' at 72ºC. We used the $2^{-\Delta\Delta Ct}$ method (Livak method) for relative quantification of gene expression. β-Actin was utilized as the housekeeping gene. The following list of primers was used: β-Actin F: 5'-GCGAGAAGATGACCCAGATC-3' , R: 5'-GGATAGCACAGCCTGGATAG-3'; COX-2 F: 5'-CACCCATGTCAAAACCGAGG-3' , R: 5'-CCGGTGTTGAGCAGTTTTCTC-3'; hTERT F:5'-GTCGAGCTGCTCAGGTCTT-3', R: 5'-AGTGCTGTCTGATTCCAATGCTT-3'; MDR1, F:5'-TGACAGCTACAGCACGGAAG-3' , R:5'-TCTTCACCTCCAGGCTCAGT-3'.

COX-2 activity assay

CaCo-2 and Hep3B cytosols were extracted as previously described [Scarpa ES et al., 2017], but using the COX lysis buffer: 100 mM Tris/HCl pH 7.8, 1 mM EDTA. The protein concentration was assessed through the Bradford assay (Biorad Laboratories, Hercules, USA), measuring the absorbance values at λ=595 nm. COX-2 activity was assessed through the COX Activity Assay Kit (Cayman chemical, USA) according to the manufacturer's instructions. 150 µg of proteins were incubated for 10 min at 25 C with COX assay buffer (100 mM Tris/HCl pH 8.0) (CTRL) or COX-1 inhibitor SC-560, or SC-560 and 110 µM AVNs Mix, or SC-560 and 110 µM AVN 2c-S. The samples were then incubated with 210 µM arachidonic acid and the colorimetric substrate TMPD. The absorbance of the samples was measured at λ=590 nm, using a spectrophotometer. The

following formula was used to calculate the enzymatic activity: COX Activity = ($\Delta A_{590\ nm}$/10 min/0.00826 μM^{-1})x1.0 ml/ml of cell cytosol/2.

Statistical analysis

Data were expressed as mean ± SD. The ORAC assay values were the average of 8 independent determinations for each dilution used. Statistical significance was assessed by one-way ANOVA, using Past 3 Software. The results were considered statistically significant when $p < 0.05$.

Results

Syntheses and purification of avenanthramides

The chemical synthesis of AVN 2c-S, 2f-S and 2p-S was performed by adapting the protocol of Wise et al. (2011) with minor modifications. The NMR and MS data of the individual synthetized AVNs were the followings:

1) (*E*)-5-hydroxy-2-(3-(4-hydroxyphenyl)acrylamido)benzoic acid **2p**-S

MS (ESI^-) m/z 298 $[M - H]^-$; ^1H NMR (400 MHz, CD_3OD) δ 6.52 (d, *J* = 15.6 Hz, 1H), 6.82 (d, *J* = 8.6 Hz, 2H), 7.02 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.48 (d, *J* = 8.6 Hz, 2H), 7.51 (d, *J* = 3.0 Hz, 1H), 7.57 (d, *J* = 15.6 Hz, 1H), 8.45 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 116.8, 118.2, 119.3, 120.6, 122.0, 123.5, 127.5, 130.9, 134.8, 143.0, 154.2, 160.9, 166.8, 171.2.

2) (*E*)-2-(3-(3,4-dihydroxyphenyl)acrylamido)-5-hydroxybenzoic acid **2c-S**

MS (ESI^-) m/z 314 $[M - H]^-$; ^1H NMR (400 MHz, CD_3OD) δ 6.45 (d, *J* = 15.6 Hz, 1H), 6.79 (d, *J* = 8.1 Hz, 1H), 6.97 (dd, *J* = 8.2, 2.1 Hz, 1H), 7.02 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.07 (d, *J* = 2.1 Hz, 1H), 7.50 (d, *J* = 15.6 Hz, 1H), 7.51 (d, *J* = 3.0 Hz, 1H), 8.45 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 115.1, 116.5, 118.2, 119.3, 119.4, 122.0, 122.5, 123.4, 128.1, 134.8, 143.4, 146.8, 149.1, 154.1, 166.7, 171.3.

3) (*E*)-5-hydroxy-2-(3-(4-hydroxy-3-methoxyphenyl)acrylamido)benzoic acid **2f-S**

MS (ESI$^-$) m/z 328 [M − H]$^-$; ^{1}H NMR (400 MHz, CD_3OD) δ 3.92 (s, 3H), 6.56 (d, *J* = 15.6 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 7.03 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.09 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.23 (d, *J* = 1.9 Hz, 1H), 7.51 (d, *J* = 3.0 Hz, 1H), 7.56 (d, *J* = 15.6 Hz, 1H), 8.46 (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 56.5, 111.6, 116.5, 118.2, 119.3, 119.6, 122.0, 123.5, 123.8, 128.1, 134.8, 143.3, 149.4, 150.2, 154.2, 166.7, 171.2.

In the natural AVNs mix, purified through column chromatography, the relative ratios of the AVN forms were the following: 2p (37%), 2c (35%) and 2f (8%), accounting for 80% of the mix. A 20% of minor AVN forms (Lp and Lf) was also present. Any attempt to increase the purity of AVN 2p, 2c, 2f would cause the loss of one of them and changes in the relative proportions.

ORAC evaluation of synthetic AVNs

Figure 2 shows the ORAC values of synthetic AVNs, with the AVN 2c-S resulting the most active antioxidant towards the peroxyl radical. The ORAC value increased with the dilution, as the synthetic AVNs reached the maximum ORAC value at 1000 fold dilution, with the 2c-S ORAC significantly higher than that of the 2f-S or 2p-S (**Figure 2**). ORAC value of the natural AVN mix was previously detected [Scarpa ES et al., 2017].

Antiproliferative effects of AVN mix and synthetic AVNs

The antiproliferative effects of synthetic AVNs and natural AVN mix were evaluated on CaCo-2 colon cancer cells and Hep3B liver cancer cells. The IC50 values for both cancer cell lines, during the time course of 24, 48 and 72h, are listed in **Table 1**. As the IC_{50} values in Hep3B cells were significantly lower than the CaCo-2 cells IC_{50} values, it emerged that the hepatocarcinoma cell line was more sensitive to the antiproliferative effect of AVNs than the colorectal cancer cell line. In addition, in the CaCo-2 cells, the cytotoxic effect reaches its maximum after 48h and persisted at

72h, whereas in Hep3B the greatest cytotoxic effect was achieved after 24h treatments and persisted during the time course.

The synthetic AVN mix was set up in order to simulate the relative percentage of AVNs found in the natural AVN mix (35% 2c; 37% 2p; 8% 2f). The cytotoxic activity of the synthetic mixture provided a IC_{50} value of 120±14 µM in Hep3B, a value not significantly different from the IC_{50} of the natural AVN mix (p=0.84). In CaCo-2 cancer cells, the IC_{50} of the synthetic AVN mix was 280±30 µM, a value higher than that of the natural AVN mix (p=0.003).

Modulation of the caspase activities by AVN mix, 2c-S, 2f-S and 2p-S

We then evaluated the ability of AVNs mix and synthetic AVNs to activate caspase 3, 8 and 2 after 24h treatments, on the basis of a previous report [Berger A. et al., 2011].

The natural AVNs mix and the individual synthetic AVNs induced a significant increase in the activity levels of caspase 3 and 8 in both CaCo-2 and Hep3B cancer cells (**Table 2**). In CaCo-2 cells, the increase of caspase 3 activity exerted by AVN 2c-S was greater than the increase exerted by AVNs mix, AVN 2f-S and AVN 2p-S **(Table 2)**; the increase of caspase 8 due to AVN 2c-S was still higher than the activation induced by AVN 2f-S and 2p-S, but not significantly different from the effect induced by AVN mix (**Table 2**).

In Hep3B cells, the synthetic and the natural AVNs induced a significant increase in caspase 3 and caspase 8 activity, with no significant differences among them (**Table 2**).

As caspase 2 can cooperate with caspase 8 to increase the activity levels of caspase 3 [Lin CF et al., 2004], we also evaluated the activity levels of this enzyme. **Table 2** shows that AVN mix and AVN 2c-S treatments were able to significantly activate the caspase 2 in both CaCo-2 and Hep3B cells (**Table 2**).

Cellular antioxidant activity of synthetic AVNs

The DCFH-DA assay was performed with the natural AVN mix and the synthetic AVNs in CaCo-2 cells. Results show that AVN 2c-S, 2f-S and 2p-S are all able to enter inside the cancer cells and deplete the H_2O_2-mediated oxidative stress (**Figure 1SA**). Quantitative data analysis did not reveal any difference among AVNs mix and AVN 2c-S, 2f-S, 2p-S after 24h treatment (**Figure 1SB**).

Downregulation of hTERT, COX-2 and MDR1 expression

The ability of natural AVN mix and synthetic AVN 2c-S to inhibit the pro-survival pathways in the two cancer cell lines was assessed, through the analysis of the expression levels of hTERT, COX-2 and MDR1 genes (**Figure 3**).

In analogy with a previous study on other polyphenols [Thelen, P. et al., 2005], a remarkable downregulation of the expression levels of the gene hTERT, exerted by both AVN mix and AVN 2c-S on CaCo-2 (**Figure 3a**) and Hep3B (**Figure 3b**) cancer cells was observed.

In line with our previous results [Farabegoli, F. et al., 2017], we showed that both AVNs mix and AVN 2c-S were able to reduce the COX-2 mRNA levels in CaCo-2 cancer cells (**Figure 3c**); otherwise only AVN mix was able to downregulate COX-2 expression in Hep3B cells (**Figure 3d**).

In the light of a previous report [Eid SY ey al., 2013], we showed a downregulation of the expression levels of MDR1 by both AVN mix and AVN 2c-S in CaCo-2 cells (**Figure 3e**), while only AVN mix treatment reduced the MDR1 mRNA levels in Hep3B cells (**Figure 3f**).

COX-2 activity inhibition

In the light of a previous report [Sun, G. et al., 2017], we evaluated the AVN-mediated modulation of the activity levels of COX-2. COX-2 isoform accounted for 92.0±4.2 % and 94.5±1.5 % of the COX activity in CaCo-2 and Hep3B cells, respectively. AVN mix and AVN 2c-S were able to reduce COX-2 activity by 50.1±10.3 % and 19.5±4.9 %, respectively (**Figure 4a**), whereas in Hep3B cells, the AVN mix reduced the COX-2 activity by 27.0±5.5 %, while the AVN 2c-S did not exert any significant activity inhibition (**Figure 4b**).

Discussion

The synthesis of AVN 2c-S, 2f-S, 2p-S was made in our laboratory following a published protocol [Wise et al., 2011], with few modifications and a slight yield increase. The three synthetic AVNs showed extremely high ORAC values, but the AVN 2c-S was able to exploit the highest ORAC in comparison with the 2p-S, 2f-S. The 100-1000 dilution factors increased about two folds the ORAC values of the synthetic AVNs, likely because at lower dilutions, the single synthetic AVNs aggregate by means of weak intermolecular bonds, thus limiting their reducing power, as a consequence of matrix interactions and/or steric hindrance [Huang, D. et al., 2005]. At the highest dilution, the molecules exploited their whole antioxidant capacity. Therefore, AVNs resulted powerful antioxidants, able to exert their reducing power inside the cells.

The DCFH-DA assay showed the permeability of the synthetized AVN across the cancer cell membrane, as well as their intracellular antioxidant activity. The synthetic and natural AVNs exploited similar intracellular antioxidant efficiency after 24h incubation time. As it has been demonstrated that AVNs 2p and 2c possess higher cell permeability and bioavailability than the AVN 2f [Chen CYO et al., 2007], it would be possible that after shorter incubation times, some differences in the permeability of the synthetized AVNs could be detected, but this aspect is out of the objectives of the present work.

The three synthetized AVNs, as well as the natural AVN mix, were cytotoxic against both CaCo-2 and Hep3B cancer cells, with few differences between the two cell lines and among the AVN forms. In CaCo-2 cells, our results showed that the cytotoxicity of AVN 2c-S was higher than that of 2f-S, 2p-S and natural AVN mix, while in the Hep3B cells, no significant difference among the three synthetic forms and AVN mix was observed. However, the AVNs antiproliferative effect was higher in Hep3B than in CaCo-2 cancer cells, as indicated by the lower IC_{50} of synthetic and natural

AVN mix measured in Hep3B cells in comparison with CaCo-2 cells. Differences in the genetic background and efficiency of anti-apoptotic mechanisms between the two cancer cell lines probably account for this discrepancy. Experiments aimed to simulate the natural AVN mixture, with the same relative proportions of synthetic AVNs, confirmed again the higher vulnerability of the Hep3B cells, when compared with CaCo-2 cells. It is worthy to remember that Hep3B possess a relevant genetic resistance to anticancer agents, acquired through an overactivation of the MDR mechanisms [Gambari et al., 2014]. Therefore, molecules, such as AVNs, able to target the proliferative capacity of these cells are highly profitable, especially when they can target cancer cells with no damage to normal human cells [Scarpa ES et al., 2017].

The synthetic AVNs and the natural AVN mix were able to activate the extrinsic apoptotic pathway, based on the induction of the caspase 8 and 3, as well as the caspase 2, whose AVN-mediated activation was here demonstrated for the first time. The contribution of the caspase 2 is triggered when a phytochemical is able to interact with the intracellular network represented by the PIDDosome, the molecular complex that leads to the maturation and activation of caspase 2 [Puccini J et al., 2013].

The activation of the network of the caspases is only one aspect accounting for the cytotoxicity of the synthetic and natural AVNs on the two cancer cell lines. A strengthening of the pro-apoptotic effect of the AVNs was also provided through the downregulation of some pro-survival markers. Our results show a remarkable downregulation of hTERT expression by AVN mix and AVN 2c-S treatments. hTERT is the gene that encodes for the telomerase and nutritional phytochemicals, like AVNs, able to target hTERT, could be utilized in molecular cocktails for antitumor therapies [Colomer R et al., 2017]. Our results also show that the AVN treatments reduced the expression levels of other two pro-survival genes: COX-2 and MDR1. Surprisingly, a significant reduction in both cell lines was obtained by the AVN mix only, whereas AVN 2c-S was able to deplete the

expression levels of COX-2 and MDR1.in CaCo-2, but not in the Hep3B cell line. The failure of AVN 2c-S to downregulate the pro-survival markers COX-2 and MDR1 in Hep3B cells needs to be elucidated. However, the lack of reduction of both COX-2 and MDR1 expression levels in Hep3B cells, treated with AVN 2c-S, is in agreement with the discovery that these two molecular markers are linked [He L. et al., 2015].

As in several cancers, including adenocarcinomas and hepatocarcinomas, elevated COX-2 expression and activity levels were correlated with a poor response to therapy [Diab S et al., 2015], the reduction of COX-2 activity levels in CaCo-2 and Hep3B cancer cells due to the natural AVN mix, strengthen the role of AVNs as effective chemopreventive agents. On this basis, the AVN mix is more efficient than the individual AVN 2c-S, thus indicating that probably some synergistic effects take place in the AVN mix among the 2c, 2f and 2p forms. The network of the intracellular pathways triggered by the AVNs is complex and requires further investigation.

To the effects of the AVNs on the caspase activation and reduction of expression levels of hTERT, COX-2 and MDR1, we must add the already demonstrated downregulation of HIF1A and VEGFA genes [Scarpa ES et al., 2017], which leads to the depletion of antioxidant defenses and survival abilities of cancer cells. The inhibition of the transcriptional activity of the pro-inflammatory and pro-carcinogenetic marker NFkB demonstrated by others [Koenig RT et al., 2016] adds a new element, useful to clarify this complex network. We already demonstrated that AVNs were not able to downregulate BIRC5 expression [Scarpa ES et al., 2017], but other unknown targets of AVNs remain to be discovered.

In conclusion, the synthetic AVNs 2p, 2f and 2c and the natural AVNs mix possess pleiotropic anticancer properties, with differences in efficacy in two neoplastic cell lines, characterized by different histopathology. The natural AVN mix appears as the strongest anticancer cocktail, whereas, among the synthetic AVNs, the 2c-S results the most efficient one.

The possibility to synthetize on a large scale the individual AVNs will provide the opportunity to perform further investigations aimed to combine the AVNs in several different mixtures, changing the relative proportions of the three AVN forms in order to obtain the complete characterization of the AVNs molecular targets.

A mixture of synthetic AVNs similar to that found in oat or in oat-derived extracts, could result more effective in chemoprevention and anti-inflammatory therapy than a single AVN. The anti-inflammatory effect of AVNs is well ascertained and it was confirmed through inhibition of COX-2 enzyme activity and reduction of its mRNA levels.

As the AVNs are present in food, they possess low toxicity for humans and their possible use in new therapies will provide weak, if any, side effects. In the case of development of new foods, fortified with AVNs, their utility for both chemoprevention and treatment of chronic inflammatory states has stepped forward and in vivo experiments represent the future perspectives for AVN treatments.

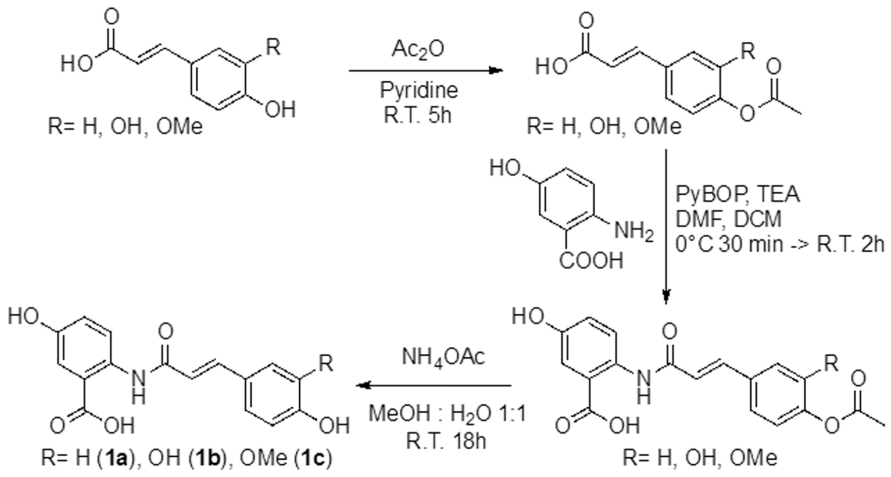

Figure 1. Scheme of the reactions occurring in the syntheses of avenanthramides.

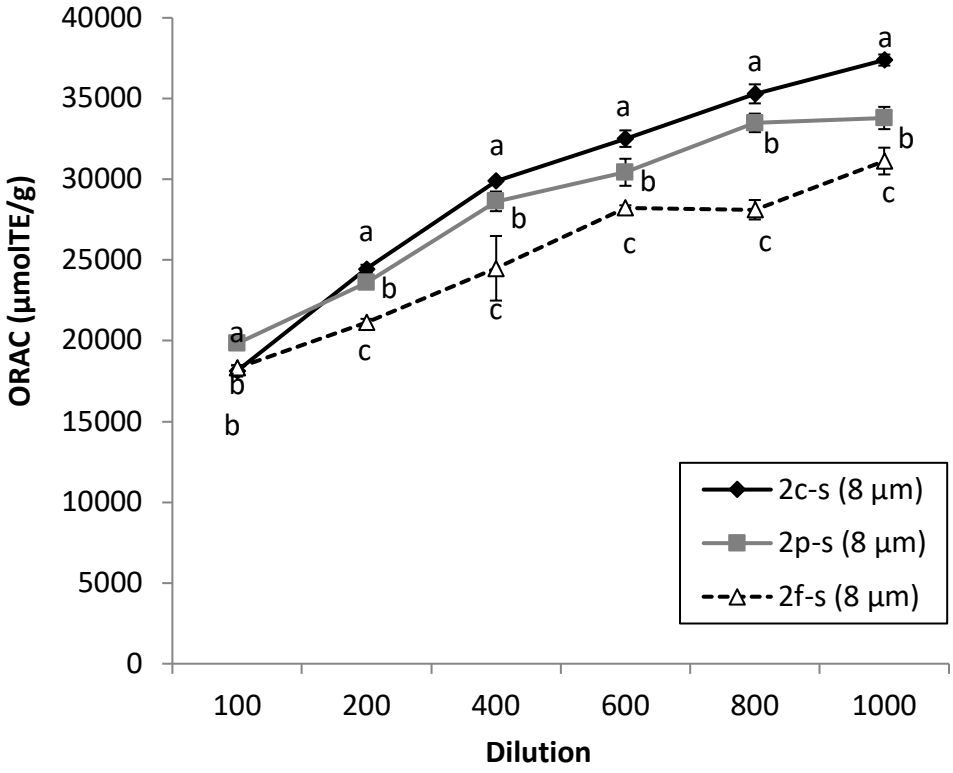
40000
35000
30000
25000
20000
15000
10000
5000
0
ORAC (µmolTE/g)
100
200
400
600
800
1000
Dilution
2c-s (8 µm)
2p-s (8 µm)
2f-s (8 µm)

Figure 2, (n=8).

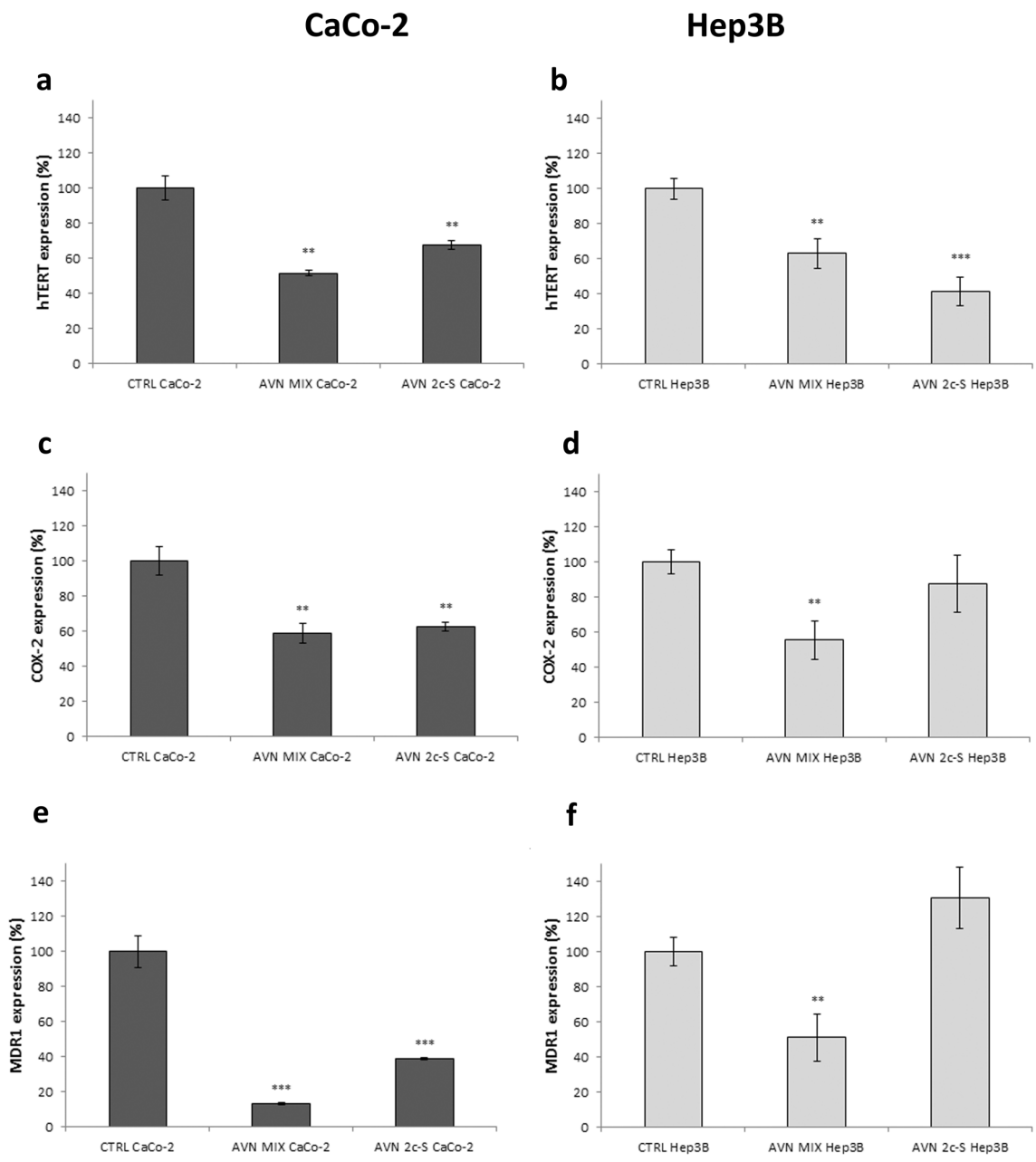

Figure 3. Evaluation of the effects of AVN mix and AVN 2c-S on hTERT, COX-2 and MDR1 expression levels in CaCo-2 (a, c, e) and Hep3B (b, d, f) cancer cells. hTERT (a, b), COX-2 (c, d), MDR1 (e, f) mRNA levels were measured and normalized to the mRNA levels of the housekeeping gene β-actin. Results obtained from untreated cells (CTRL) and from CaCo-2 and Hep3B cells treated with AVN mix (110 μM) or AVN 2c-S (110 μM) for 24h, are shown. Two replicate experiments with three samples analyzed for each replicate (n=6) were performed. **$p<0.01$, ***$p<0.001$

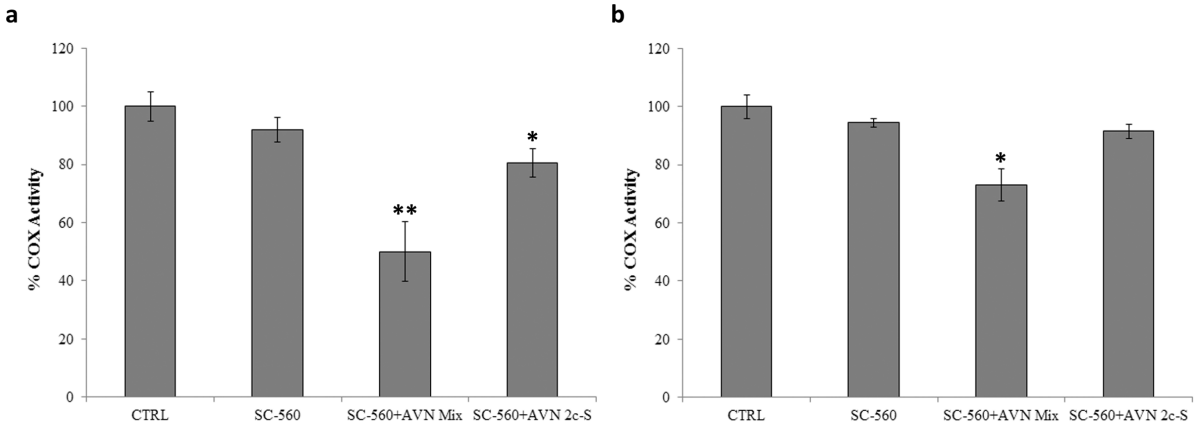

Figure 4. Evaluation of COX activity levels, modulated by AVN mix and AVN 2c-S in the cytosols extracted from CaCo-2 (a) and from Hep3B (b) cancer cells. Cytosols were untreated (CTRL) or incubated with COX-1 inhibitor SC-560 alone or with COX-1 inhibitor and 110 µM AVN Mix or with COX-1 inhibitor and 110 µM AVN 2c-S for 10 min at RT. Three replicate experiments (n=3) were performed. *p<0.05, **p<0.01

Table 1. IC_{50} values

Table 2. Caspase 3, 8 and 2 activities

FIGURE 1 Supplementary. a) Representative images of DCFH-DA assay in CaCo-2 cells treated with natural and synthetic AVNs before H_2O_2 treatment. CTRL, untreated cells; H2O2, cells treated with 1 mM H2O2 for 1h; AVN MIX+H2O2, cells treated with 110 uM AVN mix for 24h and then with 1 mM H2O2 for 1h; AVN 2c-S+H2O2, cells treated with 110 uM AVN 2c-S for 24h and then with 1 mM H2O2 for 1h; AVN 2f-S+H2O2, cells treated with 110 uM AVN 2f-S for 24h and then with 1 mM H2O2 for 1h; AVN 2p-S+H2O2, cells treated with 110 uM AVN 2p-S for 24h and then with 1 mM H2O2 for 1h. **b)** Three fields per sample were analyzed and each time at least 100 cells were counted; two independent experiments were performed (n=6) and results were expressed as % DCF fluorescent positive cells versus control; ***p<0.001

References……..