Bioorganic & Medicinal Chemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Combined dual effect of modulation of human neutrophils' oxidative burst and inhibition of colon cancer cells proliferation by hydroxycinnamic acid derivatives

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ARTICLE INFO

Article history:
Received 30 March 2016
Revised 26 May 2016
Accepted 29 May 2016
Available online xxxx

Keywords:
Hydroxycinnamic acid derivatives
Synthesis
Human neutrophils
Oxidative burst
Colon cancer cells
Antiproliferative

ABSTRACT

Colon cancer is one of the most incident cancers in the Western World. While both genetic and epigenetic factors may contribute to the development of colon cancer, it is known that chronic inflammation associated to excessive production of reactive oxygen and nitrogen species by phagocytes may ultimately initiate the multistep process of colon cancer development. Phenolic compounds, which reveal antioxidant and antiproliferative activities in colon cancer cells, can be a good approach to surpass this problem. In this work, hydroxycinnamic amides and the respective acid precursors were tested in vitro for their capacity to modulate human neutrophils' oxidative burst and simultaneously to inhibit growth of colon cancer cells. A phenolic amide derivative, caffeic acid hexylamide (CAHA) (**4**) was found to be the most active compound in both assays, inhibiting human neutrophils' oxidative burst, restraining the inflammatory process, inhibiting growth of colon cancer cells and triggering mitochondrial dysfunction that leads cancer cells to apoptosis. Altogether, these achievements can contribute to the understanding of the relationship between antioxidant and anticancer activities and based on the structure–activity relationships (SAR) established can be the starting point to find more effective phenolic compounds as anticancer agents.

1. Introduction

Colon cancer is one of the most incident cancers in the Western World[1] and nowadays it is also spreading into Asian countries, probably due to the adoption of Western diet. In spite of relevant improvement in survival over the past decade, a significant number of patients relapse after surgical and conventional therapies and do not respond to metastatic cancer treatment.[2,3] On the other hand, it is well known that cytotoxic drugs used in chemotherapy protocols have several adverse effects namely the weakening of patients' immune system, also destroying peripheral blood mononuclear cells, which are critical components of the immune system to fight opportunistic infections as well as cancer cells. Taking this knowledge into account, the search for new therapeutic options for the chemoprevention and/or the treatment of colon cancer is a matter of interest.

In addition, it has been assumed that excessive production of reactive oxygen (ROS) and nitrogen (RNS) species by phagocytes, namely neutrophils, may lead to chronic inflammation and ultimately initiate the multistage process of colon cancer development.[4,5] Further, it has been described that ROS are involved, not only in the initiation of the carcinogenesis process, but also in cancer promotion and progression.[6,7] In particular, the inflammatory bowel disease, namely the ulcerative colitis, has been linked to an increased risk of colorectal cancer being the oxidative reactions an important part of the inflammatory response.[8,9]

A large amount of scientific evidence reported in the literature suggests that phenolic compounds present in diet or consumed

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http://dx.doi.org/10.1016/j.bmc.2016.05.065
0968-0896/

alone can act as chemopreventive and/or chemotherapeutic agents.[10] Among these, hydroxycinnamic acid derivatives constitute a major group of antioxidant compounds with inhibitory activity in proliferation of several cancer cell lines. Particularly, caffeic acid (CA) showed a protective effect on paclitaxel induced antiproliferation and apoptosis of lung cancer cells[11] however it also presented antiproliferative effects against colon,[12,13] fibrosarcoma,[14] breast,[15,16] cervical,[16] liver,[15,17] and leukemia cancer cells.[18] Ferulic acid (FA) is described as an antiproliferative agent against breast and liver cancer cells[15] and revealed to delay the cell cycle progression, specifically in the S and G2/M phases of colon cancer cells.[19] 3,4,5-Trihydroxycinnamic acid (OHCA) showed antiproliferative activities in cervical, colon, prostate and oral cavity cancer cell lines.[20] Recently, new lipophilic caffeic and ferulic acid derivatives were synthesized and their cytotoxicity was compared with that of the parent compounds showing increased cytotoxicity towards breast cancer cell lines. These results indicated that the new compounds inhibited cell proliferation and induced cell cycle alterations and cell death in the referred cancer cells.[21]

Based on the above-mentioned considerations, we aimed to find new and more effective agents suitable for chemoprevention and/or chemotherapeutic purposes against colon cancer which will be able to simultaneously modulate human neutrophils' oxidative burst, restraining the inflammatory process, and to inhibit growth of colon cancer cells. For this purpose, *n*-hexylamide derivatives of caffeic, ferulic and 3,4,5-tryhydroxycinnamic acids with superior lipophilicity and consequently with improved ability to cross cell membranes were synthesized (Scheme 1). Subsequently, they were screened along with their parent acids, to test their anti-inflammatory activity against human neutrophils' oxidative burst as well as in terms of cytotoxicity, on two colon adenocarcinoma cell lines with different genetic background and origin localization, WiDr (rectosigmoid) and C2BBe1 (descending colon). Finally, some structure–activity considerations were inferred.

2. Materials and methods

2.1. Chemistry

Reactions were controlled by thin layer chromatography (TLC) using silica gel 60 F254 plates. Column chromatography was performed using silica gel 60 (0.063–0.200 mm). Melting points (MPs) were determined on a Reichert Thermopan hot block apparatus and were not corrected. IR spectra were recorded on a Jasco 420 FT/IR spectrometer. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 600 MHz and 150 MHz, respectively on a Varian Unity 600. Chemical shifts were recorded in δ values in parts per million (ppm) downfield from tetramethylsilane as an internal standard. All *J* values are given in Hz. Caffeic (**1**) and ferulic (**2**) acids were purchased from Sigma–Aldrich (Schnelldorf, Germany) and 3,4,5-trihydroxycinnamic acid (**3**) to Apin Chemicals Limited (Abingdon, Oxon, United Kingdom). Reagents and solvents were used as obtained from suppliers without further purification.

2.2. General procedure to obtain the cinnamic acid hexylamides 4, 5 and 6

To synthesize the amides CAHA (**4**), FAHA (**5**) and OHCAHA (**6**), cinnamic acids CA (**1**), FA (**2**) and OHCA (**3**), respectively (Scheme 1) were dissolved in dimethylformamide (DMF) and triethylamine (TEA). The solution was then cooled in an ice-water bath and hexylamine was added, followed by a solution of (benzotriazol-1-yloxy) tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in dichloromethane. The mixture was stirred at 0 °C for 30 min and then at room temperature for specific periods of time. Dichloromethane was removed under reduced pressure and the remaining solution was diluted with water (100 mL). The mixture was then extracted with ethyl acetate (2 × 100 mL). The extracts were washed with 1 N HCl (2 × 100 mL), water (2 × 100 mL), $NaHCO_3$ 5% (3 × 100 mL) and finally with water (2 × 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated. The obtained residues were purified by column chromatography yielding the corresponding hexylamides (**4**, **5** and **6**).

2.2.1. *N*-Hexyl-3-(3,4-dihydroxyphenyl)-2-propenamide (4)

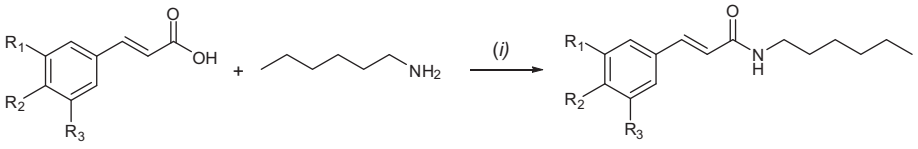
As described before.[22]

2.2.2. *N*-Hexyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenamide (5)

As described before.[22]

2.2.3. *N*-Hexyl-3-(3,4,5-trihydroxyphenyl)-2-propenamide (6)

Compound **3** (250.0 mg, 1.27 mmol); DMF (2.9 mL); TEA (0.18 mL); hexylamine (0.17 mL, 1.27 mmol); BOP (561.7 mg, 1.27 mmol); CH_2Cl_2 (3 mL). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 5 h 30 min. The residue obtained after work-up was purified by silica gel column chromatography (hexane/ethyl acetate) giving the pure compound **6** in 39% yield. $Mp_{(hexane/ethyl\ acetate)}$ 93–97 °C. IR (NaCl plates, $CHCl_3$) ν_{max} cm^{-1}: 3267 (N—H), 1642 (C=O), 1315 (C—O phenolic alcohol). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.85 (3H, m, $-CH_3$), 1.28 (6H, m, $-CH_2(3'–5')$), 1.41 (2H, m, $-CH_2(2')$), 3.12 (2H, dd, J = 12.9, J = 6.9, $-CH_2(1')$), 6.26 (1H, d, J = 15.6, –CH (α)), 6.47 (2H, s, –CH(2 and 6)), 7.11 (1H, d, J = 15.6, –CH(β)),



(**1**) $R_1 = R_2 = OH$; $R_3 = H$

(**2**) $R_1 = OCH_3$; $R_2 = OH$; $R_3 = H$

(**3**) $R_1 = R_2 = R_3 = OH$

(**4**) $R_1 = R_2 = OH$; $R_3 = H$

(**5**) $R_1 = OCH_3$; $R_2 = OH$; $R_3 = H$

(**6**) $R_1 = R_2 = R_3 = OH$

(**1**) caffeic acid (CA)
(**2**) ferulic acid (FA)
(**3**) 3,4,5-trihydroxycinnamic acid (OHCA)
(**4**) caffeic acid hexylamide (CAHA)
(**5**) ferulic acid hexylamide (FAHA)
(**6**) 3,4,5-trihydroxycinnamic acid hexylamide (OHCAHA)

Scheme 1. Synthesis and structures of hydroxycinnamic acid *n*-hexylamide derivatives. Reagents and conditions: (i) dimethylformamide (DMF), triethylamine (TEA), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), dichloromethane, rt.

Please cite this article in press as: Tavares-da-Silva, E. J.; et al. *Bioorg. Med. Chem.* (2016), http://dx.doi.org/10.1016/j.bmc.2016.05.065

7.94 (1H, t, J = 5.7, –NH), 8.15 (1H, s, –OH(4)), 9.03 (2H, s, –OH(3 and 5)). ^{13}C NMR (150 MHz, DMSO-d_6) δ: 13.9 (CH_3), 21.9, 26.1, 29.1, 30.9 (C-2′)–(C-5′), 38.5 (C-1′), 106.6 (C-2 and C-6), 118.6 (C-α), 125.3 (C-1), 135.0 (C-4), 139.1 (C-β), 146.0 (C-3 and C-5), 165.1 (C = O).

2.3. Isolation of human neutrophils

Neutrophils were isolated from blood collected from human volunteers, following approval by the Ethical Committee of Hospital Geral de Santo António, CHP, Porto, and all procedures comply with Helsinki Declaration. The design and execution of the experiment were thoroughly explained to the participants, and informed consent was obtained for blood collection. The following assays were carried out using blood from $n \geqslant 8$ individuals. Venous blood was collected by antecubital venipuncture, from each human healthy volunteer into vacuum tubes with K_3EDTA. The isolation of human neutrophils was performed by the density gradient centrifugation method as previously reported.[23] Tris–glucose (25 mM Tris, 1.26 mM $CaCl_2{\cdot}2H_2O$, 5.37 mM KCl, 0.81 mM $MgSO_4$, 140 mM NaCl, and 5.55 mM D-Glucose) was the incubation media used in the evaluation of neutrophils' oxidative burst, as previously recommended.[24] All of the reagents used in isolation of human neutrophils were [Sigma–Aldrich Co. LLC (St. Louis, USA)].

2.4. Human neutrophils' viability

Cell viability was determined by the trypan blue exclusion assay. Neutrophils were incubated with all the tested compounds (at the maximum concentration tested, 200 μM) for 1 h at 37 °C. Twenty microliter of neutrophil suspension were added to an equal volume of trypan blue solution 0.4% (Sigma–Aldrich Co., St. Louis, USA) in a microtube and gently mixed. After 2 min on ice, neutrophil number and viability (viable cells excluding trypan blue) were counted. Assays were performed in triplicate.

2.5. Measurement of human neutrophils' oxidative burst

The chemiluminescent probe luminol has been thoroughly studied and used for monitoring the production of reactive species by neutrophils, namely the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), hypochlorous acid (HOCl), nitric oxide ($^{\cdot}NO$) and peroxynitrite anion ($ONOO^-$).[25] The measurement of neutrophils' oxidative burst was undertaken by chemiluminescence, by monitoring reactive species-induced oxidation of luminol [Fluka Chemie GmbH (Steinheim, Germany)] using a microplate reader (Biotek® Synergy HT). The reaction mixtures contained neutrophils (2×10^6 cells/mL) and the following reagents at the indicated final concentrations (in a final volume of 250 μL): tested compounds at various concentrations, luminol (500 μM) and phorbol myristate acetate (PMA) [Sigma–Aldrich Co. LLC (St. Louis, USA)] (160 nM). Cells were pre-incubated with luminol and the tested compounds for 5 min before the addition of PMA, and the measurements were carried out at 37 °C, under continuous soft shaking. Kinetic readings were initiated immediately after cell stimulation. Measurements were taken at the peak of the curve. This peak was observed at around 15 min. Effects are expressed as the percent inhibition of luminol oxidation. Each study corresponds to, at least, eight individual experiments, performed in triplicate in each experiment.

2.6. Colon cancer cell culture

C2BBe1 [clone of Caco-2] (ATCC® CRL-2102™) and WiDr (ATCC® CCL-218™), two colorectal adenocarcinoma cell lines, were acquired from American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco's Modified Eagle's Medium supplemented with 100 μM sodium pyruvate (Life Technologies), 5% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic (100 U/mL penicillin and 10 μg/mL streptomycin) at 37 °C with 95% air and 5% CO_2. Unless specified otherwise all cell culture reagents were [Sigma–Aldrich Co. LLC (St. Louis, USA)].

2.7. Effects of compounds on colon cancer cell protein synthesis

The effect of the compounds on colon cancer cells proliferation was evaluated using the colorimetric Sulphorhodamine B (SRB) assay. This assay relies on the uptake of the negatively charged pink aminoxanthine dye, SRB by basic amino acids in the cells, giving a measure of protein synthesis which gives information about cell proliferation.[26] For each experiment, cells were seeded in 48 multiwells in a concentration of 50,000 cells/mL. Cells were then treated with increasing concentrations of the compounds (0–200 μM) and after 24, 48, 72 and 96 h of cell incubation SRB assay was performed as described before.[27] Culture medium was removed, cells were washed twice with phosphate buffered saline (PBS), and then incubated with a frozen solution of 1% acetic acid (Panreac) in methanol for 1 h at 4 °C. The fixation solution was discarded and cells were incubated for 1 h with SRB 0.4%, at room temperature and kept in the dark. After this period, multiwells were washed with water and 10 mM Tris·NaOH (pH = 10) was added. The content of each well was transferred to a 96 multiwells plate and the absorbance was quantified at 540 nm with a reference filter of 690 nm in a spectrophotometer (Biotek® Synergy HT). Unless specified otherwise all chemicals were [Sigma–Aldrich Co. LLC (St. Louis, USA)].

2.8. Effects of CAHA (4) on colon cancer cells viability and oxidative stress

Subsequent studies were performed with the amide CAHA (**4**), given the most promising results obtained with it.

In order to perform several tests by flow cytometry, colon cancer human cell lines were treated with different CAHA (**4**) concentrations for 48 h. Based on cell proliferation results, C2BBe1 cells were treated with 22 μM and 50 μM of CAHA (**4**) and WiDr cells with 35 μM and 50 μM of CAHA (**4**).

In order to evaluate the influence of CAHA (**4**) on cell cycle, propidium iodide (PI) was used. For this purpose, a cell suspension of 1×10^6 cells for each condition was centrifuged at 1300g for 5 min and the supernatant discarded. For fixation, 200 μL of 70% ethanol was added and incubated for 30 min at 4 °C. Then, cells were washed with PBS and the supernatant discarded. Finally, it was added 200 μL of PI/RNase (Immunostep) and incubated for 15 min in dark, at room temperature. Detection was performed with an excitation wavelength of 488 nm.

Cell viability was analyzed using annexin-V/propidium iodide (AV/PI) incorporation assay. For that, 1×10^6 cells were incubated during 15 min in binding buffer with 2.5 μL of AV (Kit Immunotech) and 1 μL of PI (Kit Immunotech). Subsequently, cells were excited at a wavelength of 525 nm for AV and 640 nm for PI. In order to assess the percentage of viable, early apoptotic, late apoptotic/necrotic and necrotic cells, 10^4 events were collected.

To evaluate the effect of CAHA (**4**) on mitochondrial membrane potential ($\Delta\psi_m$), cells were labeled with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1). An estimate of $\Delta\psi_m$ is given by the ratio between the intensities of red and green fluorescence. Intracellular ROS production was analyzed using two probes: 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA, Invitrogen) to quantify intracellular peroxides and, dihydroethidium probe (DHE) to quantify intracellular $O_2^{\cdot-}$ production. Expression of intracellular reduced glutathione

(GSH) was evaluated with mercury orange. Assays were performed according to a method previously described.[28] Results are presented as mean fluorescence intensity (MFI). Unless specified otherwise all chemicals were [Sigma–Aldrich Co. LLC (St. Louis, USA)].

2.9. Statistical analysis

The results of the assays with human neutrophils were analyzed using GraphPad Prism™ (version 5.0; GraphPad Software). Results are expressed as mean ± standard error of the mean (SEM) (from at least eight individual experiments, performed in triplicate in each experiment). Cell proliferation results were analyzed and processed in software OriginPro v. 8.0, in order to determine the concentration of the compound that inhibits cell proliferation in 50% (IC_{50}), through sigmoid fitting (Boltzman function). Flow cytometry statistical analysis was accomplished using the software IBM® SPSS® Statistics, v. 20.0 (IBM Corporation, Armonk, New York, EUA). Differences between therapeutic conditions or cell lines were performed by one-factor ANOVA test followed by Bonferroni post hoc analysis for multiple comparisons. A statistical significance level of 5% was settled.

3. Results

3.1. Chemistry

The synthesis of the hexylamides **4** to **6** (Scheme 1) was performed through a single-step reaction involving the cinnamic acids **1** to **3** with hexylamine, in dimethylformamide and triethylamine, in the presence of the coupling agent BOP, at room temperature. This is a specially appropriate procedure to allow the direct amidation of α,β-unsaturated acids, leading to the desired amides in good yields (from 39% to 70%).[29]

3.2. Effect of compounds on human neutrophils' viability

Cell viability of human neutrophils was maintained over 98%, after 1 h of exposure to the tested compounds, at the maximum concentration tested, 200 μM.

3.3. Evaluation of compounds on human neutrophils' oxidative burst

The tested compounds exhibited different effects on the suppression of human neutrophils' oxidative burst, being the order of potencies: CAHA (**4**) > FAHA (**5**) > OHCA (**3**) > FA (**2**) > CA (**1**) (Fig. 1).

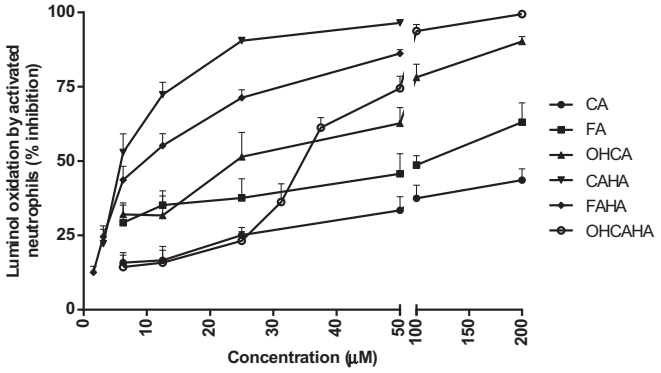


Figure 1. Effect of the compounds under study (0–200 μM) on human neutrophils' oxidative burst stimulated with phorbol myristate acetate (PMA), as measured by luminol-amplified chemiluminescence. The values are given as the mean ± SEM ($n \geqslant 8$).

As shown in Table 1, compounds CA (**1**) and FA (**2**) were noticeably less effective than the other tested compounds. Besides, it was not possible to achieve the IC_{50} value up to the highest tested concentration (200 μM) of the compound CA (**1**). It is also clear that compound CAHA (**4**) was the most effective, presenting an IC_{50} = 6.6 ± 0.8 μM, followed by FAHA (**5**) (IC_{50} = 14 ± 2 μM) and OHCA (**3**) (IC_{50} = 19 ± 4), being the effect dependent on the concentration.

3.4. Cytotoxic effect on colon cancer cell lines

The effect of the compounds on colon cancer cells proliferation was evaluated through the SRB assay, after subjecting the cells to a range of compound concentrations from 0 to 200 μM for 24, 48, 72 and 96 h of exposure. Generally, as it can be seen in Figure 2, for both cell lines, as the concentration of the compound increases, cell proliferation decreases, except for FA (**2**). Therefore, a dose-dependent response is observed, but not always accompanied by a time-dependent response (Table 2). In fact, a time-dependent inhibitory effect was observed following exposure of C2BBe1 cells to OHCA (**3**) and OHCAHA (**6**) compounds ($p = 0.003$ and $p = 0.002$, respectively).

The data obtained revealed that the parent compounds FA (**2**) and CA (**1**) (data not shown) do not have any antiproliferative effect on the colon cancer cells under study, however, the cytotoxic effect of its amide derivatives is evident. According to Figure 2 and Table 2, both cell lines revealed to be more sensitive to CAHA (**4**), with statistical differences at 48 and 72 h of incubation time for C2BBe1 cells ($p < 0.03$ and $p < 0.02$, respectively) comparing with all other compounds. Regarding the effect of OHCA (**3**), it should be noted the difference between the two cell lines response, wherein the IC_{50} values obtained for C2BBe1 cells were statistically lower than those obtained for WiDr cells ($p < 0.015$), whose lower IC_{50} value obtained was 113.7 μM after 72 h. On the other hand, it is noteworthy that WiDr cells revealed a higher sensitivity to OHCAHA (**6**) than C2BBe1 cells, with statistically significant differences for 72 h of exposure to the compound ($p = 0.009$).

Given the most promising results obtained with CAHA (**4**), subsequent studies were performed with this compound to deepen the knowledge of the cytotoxic effect.

To study CAHA (**4**) effect on cell cycle of both cell lines, PI/RNase staining assay was performed (Fig. 3). Cell cycle evaluation indicates that higher CAHA (**4**) concentrations (50 μM) induce cell cycle arrest of C2BBe1 cells on G2/M phase, with an increase of 8.8% of cells in this phase compared to control ($p = 0.013$). In contrast for WiDr cell line, higher CAHA (**4**) concentrations induce cell cycle arrest on S phase, with an increase of 9.3% of cells in this phase compared to control ($p = 0.007$). Moreover, a pre-G1 apoptotic peak was observed when WiDr cells are exposed to 50 μM of CAHA (**4**) ($p = 0.035$).

Given cell proliferation and cell cycle results, it was of great interest to study the effect of CAHA (**4**) on viability and types of induced cell death of both cell lines. So, for this purpose, the double

Table 1
Inhibition of phorbol myristate acetate (PMA)-induced neutrophils' oxidative burst by the compounds under study (IC_{50} μM, mean ± SEM)

Compound	IC_{50} (μM)
CA (**1**)	44% 200 μM*
FA (**2**)	≅200
OHCA (**3**)	19 ± 4
CAHA (**4**)	6.6 ± 0.8
FAHA (**5**)	14 ± 2
OHCAHA (**6**)	36 ± 3

* Scavenging effect (mean%) at the highest tested concentration (in superscript).

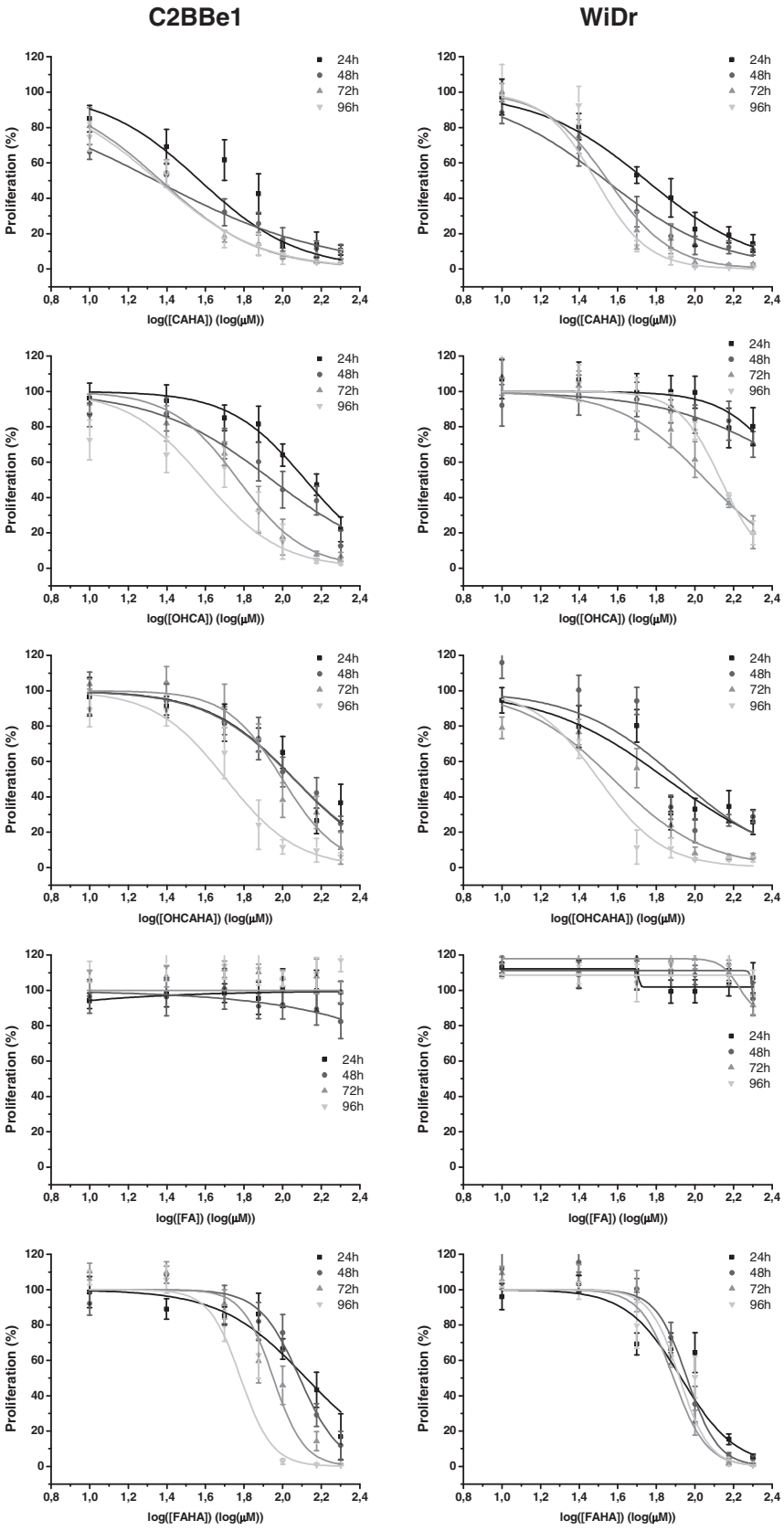


Figure 2. Effect of compounds ferulic acid (FA) (**2**), 3,4,5-trihydroxycinnamic acid (OHCA) (**3**), caffeic acid hexylamide (CAHA) (**4**), ferulic acid hexylamide (FAHA) (**5**) and 3,4,5-trihydroxycinnamic acid hexylamide (OHCAHA) (**6**) on C2BBe1 and WiDr cells proliferation. Cell proliferation was evaluated by colorimetric Sulforhodamine B assay 48 h after cells treatment. Dose–response curves were obtained after a sigmoid fitting adjust ($r^2 > 0.9$) that was performed in order to calculate the concentration needed to inhibit cell proliferation in 50% (IC_{50} values).

Table 2
Half maximal inhibitory concentrations (IC_{50} values) obtained for the two colorectal cancer cell lines after incubation with compounds ferulic acid (FA) (**2**), 3,4,5-trihydroxycinnamic acid (OHCA) (**3**), caffeic acid hexylamide (CAHA) (**4**), ferulic acid hexylamide (FAHA) (**5**) and 3,4,5-trihydroxycinnamic acid hexylamide (OHCAHA) (**6**) for 24, 48, 72 and 96 h

Cell line	Incubation time (h)	CAHA (**4**) IC_{50} (μM)	OHCA (**3**) IC_{50} (μM)	OHCAHA (**6**) IC_{50} (μM)	FA (**2**) IC_{50} (μM)	FAHA (**5**) IC_{50} (μM)
C2BBe1	24	37.3	131.2	114.8	>200	132.3
	48	22.0	87.9	114.7	>200	122.6
	72	23.4	58.9	99.8	>200	89.5
	96	22.6	39.1	50.9	>200	60.6
WiDr	24	56.1	>200	71.4	>200	84.9
	48	34.6	>200	82.4	>200	91.0
	72	35.5	113.7	37.7	>200	77.4
	96	30.8	136.4	31.7	>200	83.3

staining with AV and PI was performed. As shown in Figure 4, increasing concentrations of CAHA (**4**) induced a decrease on cell viability in both colorectal cancer cell lines under study. WiDr cells revealed to be the most sensitive to CAHA (**4**) with a decrease on cell viability of 32.8% and 43.9% compared to control, after incubation with 35 μM (IC_{50}) and 50 μM of CAHA (**4**) (p <0.001), respectively. In C2BBe1 cells it was observed a significant decrease of 11.4% on cell viability after incubation with 22 μM (IC_{50}) and a significant decrease of 14.7% with 50 μM of CAHA (**4**) (p = 0.006 and p = 0.003, respectively) comparing to control. The decrease of WiDr cells viability was accompanied by an increase of different types of cell death, mainly early apoptosis, however with no statistical significance. This result corroborates the presence of a pre-G1 apoptotic peak. In C2BBe1 cells, an increase of early apoptosis was observed with 50 μM of CAHA (**4**) (p = 0.033).

Apoptosis can be closely related to mitochondrial dysfunction, which is associated with the decrease of mitochondrial membrane potential ($\Delta\psi_m$). CAHA (**4**) also interferes with $\Delta\psi_m$ (Fig. 5-A). When both cell lines are treated with 50 μM of CAHA (**4**), concerning aggregates/monomers ratio, there is a significant increase, 1.27 ± 0.04 times, relative to control (p <0.001 for WiDr cells).

3.5. Oxidative stress on colorectal cancer cells

In order to understand whether CAHA (**4**) influences oxidative environment of colorectal cancer cells, intracellular production of $O_2^{\cdot-}$ (Fig. 5-B), peroxides (Fig. 5-C) and GSH (Fig. 5-D) was assessed by flow cytometry.

CAHA (**4**) provoked a statistical significant increase of $O_2^{\cdot-}$ production in a dose-dependent manner for both cells lines. In WiDr cells, 50 μM of CAHA (**4**) induced an increment of 2.63 ± 0.25 comparing to control (p <0.001). Regarding intracellular production of peroxides, a slight increase was observed in WiDr cells with increasing CAHA (**4**) concentrations. The production of the antioxidant peptide GSH was also influenced by the presence of CAHA (**4**). With 50 μM it was observed an increase of 1.47 ± 0.08 for C2BBe1 cells (p = 0.004).

4. Discussion

It is well known that inflammation is intrinsically involved in the promotion and progression of almost all cancers. In fact, the link between inflammation and cancers, rather than a recent concern, was noticed ~150 years ago. The involvement of the inflammatory process in the development of cancers might be a process driven by inflammatory cells.[30] At the very early stage of inflammation, neutrophils are the first cells to migrate to the inflammatory sites and defend the organism by the production of an array of reactive pro-oxidant species. As such, it was important to understand if the compounds under study were able to modulate human neutrophils' oxidative burst at non-toxic concentrations. First of all we used the trypan blue method in order to study the toxicity of the compounds under study. Our results showed that none of the tested compounds affect neutrophils viability at the maximum concentration tested (200 μM). In order to mimic the inflammatory process, we used PMA that activates protein kinase C, which results in NADPH oxidase activation with the production of $O_2^{\cdot-}$ and other reactive species through a cascade reaction.[31] Luminol was the probe used to detect the neutrophils' oxidative burst since it is able to react unspecifically with several reactive species as $O_2^{\cdot-}$, H_2O_2, HO·, HOCl, nitric oxide (·NO) and $ONOO^-$.[25] All the tested compounds demonstrated ability to decrease human neutrophils'

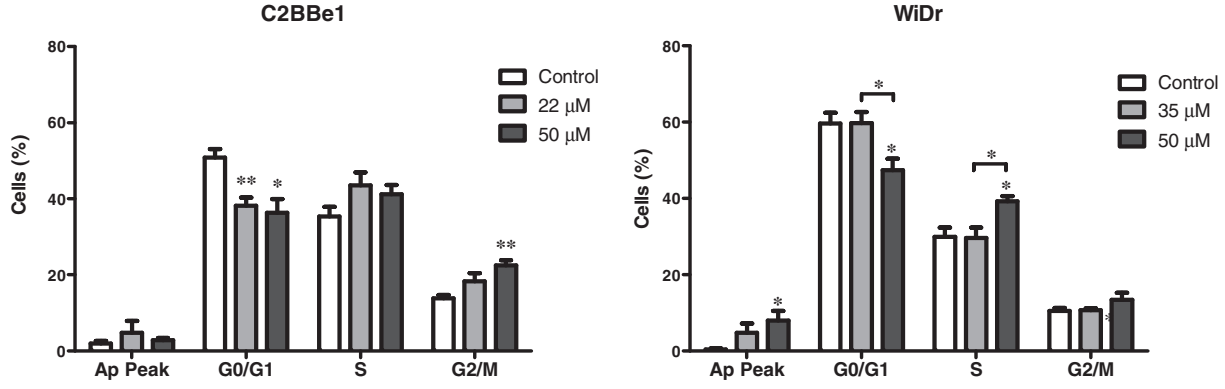


Figure 3. Cell cycle evaluation after incubation of C2BBe1 and WiDr cells with caffeic acid hexylamide (CAHA) (**4**) for 48 h. Cell cycle was evaluated by flow cytometry, using propidium iodide staining. Data represent mean ± SE of cells at each phase of cell cycle (Ap Peak, G0/G1, S and G2/M) of at least three independent experiments per cell line. Statistically significant differences are shown with $^{*}p$ <0.05, $^{**}p$ <0.01.

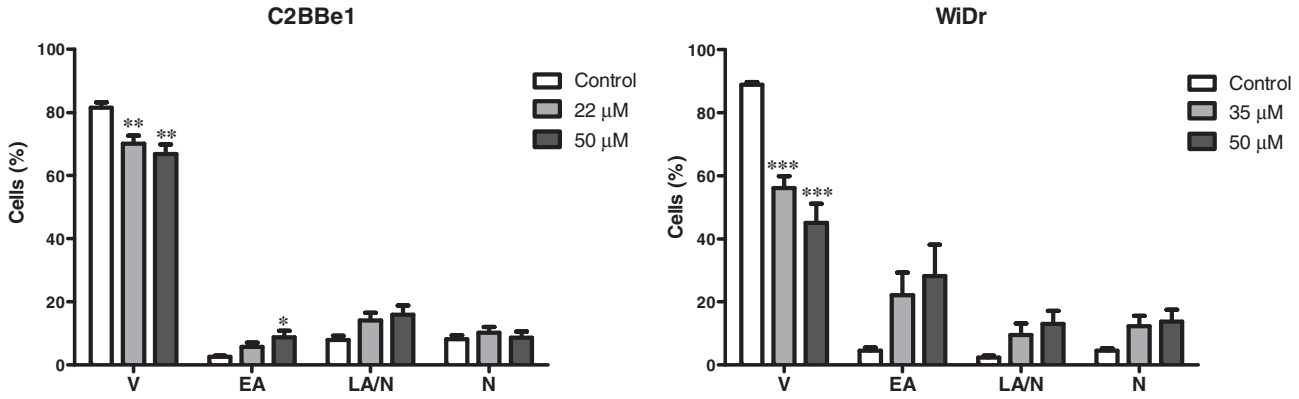


Figure 4. Cell viability analysis after incubation of C2BBe1 and WiDr cells with caffeic acid hexylamide (CAHA) (**4**) for 48 h. Cell viability and death was assessed by flow cytometry using dual-staining with annexin-V/propidium iodide. Results are expressed as the mean ± SE of viable (V), in early apoptosis (EA), in late apoptosis and/or necrosis (LA/N) and necrotic cells (N) of at least four independent experiments in duplicate. Statistically significant differences are shown with $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001.

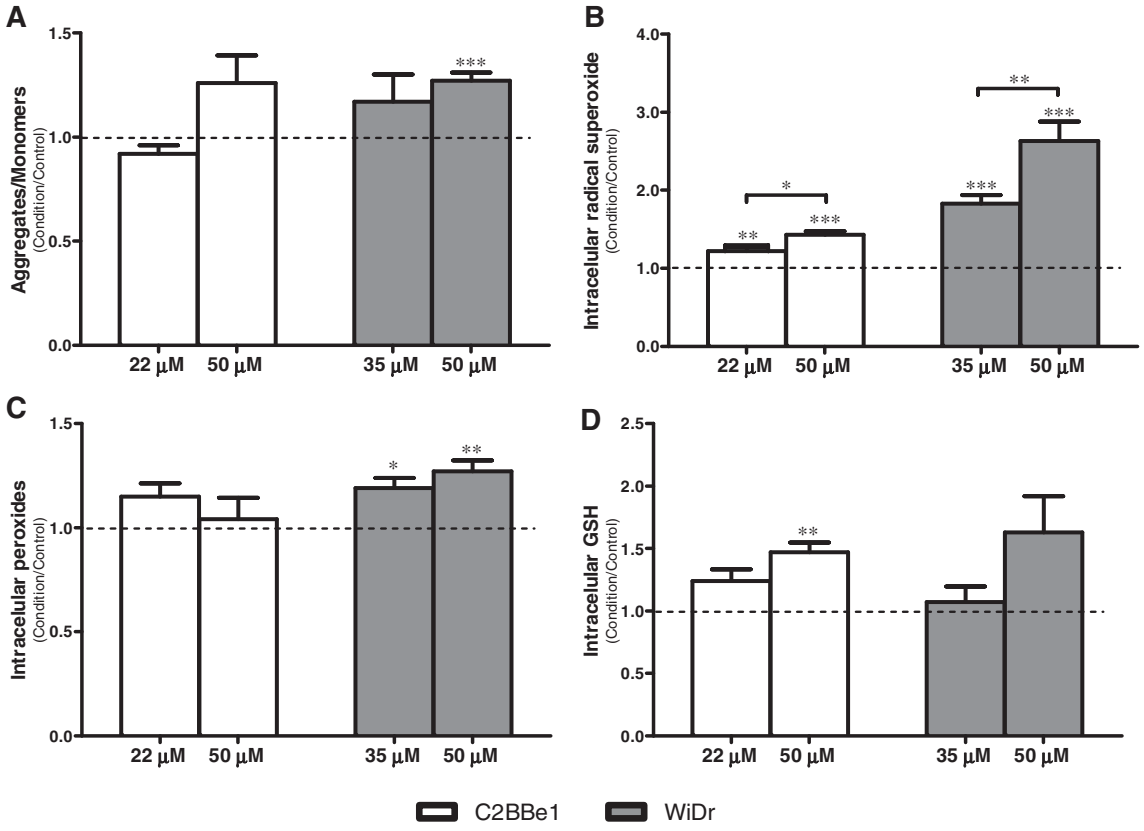


Figure 5. Analysis of mitochondrial membrane potential ($\Delta\psi_m$) (A) and intracellular production of anion radical superoxide (B), peroxides (C) and reduced glutathione (GSH) (D), after incubation of C2BBe1 and WiDr cells with caffeic acid hexylamide (CAHA) (**4**) for 48 h. These parameters were assessed by flow cytometry. Results are expressed as the mean ± SE relative to control, of at least three independent experiments in duplicate. Statistically significant differences are shown with $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001.

oxidative burst, being the most effective the amide derivative CAHA (**4**). Interestingly, as discussed below, this was also the compound that shown to be the most cytotoxic against the colon cancer cell lines. Amide derivatives CAHA (**4**) and FAHA (**5**) were more effective than the correspondent parent carboxylic acids CA (**1**) and FA (**2**) but, on the contrary, the amide derivative OHCAHA (**6**) is less effective than the parent carboxylic acid OHCA (**3**). We speculate that the superior lipophilicity of the amides **4** and **5** allows its entrance in the cell favoring its inhibitory effect on neutrophils' oxidative burst. The same argument cannot be used for the amide **6**. In this case, other forms of cell penetration rather than passive diffusion must be involved affecting the final effectiveness of carboxylic acid **3** and its amide **6**. The substitution of a hydroxyl group of the CAHA (**4**) by a methoxyl group, as in FAHA (**5**), or even an introduction of another hydroxyl group, as in OHCAHA (**6**), did not shown to be advantageous to the modulatory effect of human neutrophils' oxidative burst. The parent carboxylic acids CA (**1**) and FA (**2**) were undoubtedly the less effective in inhibiting production of reactive species by neutrophils. The inhibitory activity of the CA (**1**) and FA (**2**) on PMA-induced $O_2^{\cdot-}$ generation in human neutrophils was already reported.[32] Interestingly, the concentration of 100 μM of CA (**1**) inhibited 84% and FA (**2**) had no effect on PMA-induced $O_2^{\cdot-}$

generation. These results are in accordance with our findings. The less activity of CA (**1**) in our system can be related to the selectivity of the probe, as luminol detect several reactive species and in the reported work[32] the authors only studied the $O_2^{\cdot-}$. It is important to stress that, to the best of our knowledge, there are no reports in literature about the effect of the synthesized amide derivatives on human neutrophils.

Concerning the cytotoxicity in colon cancer cells, it was possible to infer that some of the studied compounds present effective antiproliferative activity against C2BBe1 and WiDr cells. As observed in the inhibition of human neutrophils' oxidative burst, the amide CAHA (**4**) was the most active in both colon cancer cells and amides CAHA (**4**), FAHA (**5**) and OHCAHA (**6**) (in WiDr cells) were more cytotoxic than the correspondent parent carboxylic acids CA (**1**) (data not shown), FA (**2**) and OHCA (**3**) towards the referred cell lines. Actually, the parent carboxylic acids CA (**1**) and FA (**2**) did not demonstrate any cytotoxic activity against the studied colon cancer cells. This was also observed in another study from the authors,[21] in which these compounds were tested in a breast cancer cell line (MCF-7). The explanation for this may lie in the increased lipophilicity of the amides, conferred by the additional alkyl chain, when compared to that of the parent acids, which is expected to favor the intracellular accumulation of the compounds due to their ease of crossing cell membranes. Regarding the parent carboxylic acid OHCA (**3**), and unlike the other acids, in C2BBe1 cells, after 48, 72 and 96 h, it demonstrates a better antiproliferative activity than the correspondent amide OHCAHA (**6**). Again, a similar result was observed for this compound in the inhibition assay of human neutrophils' oxidative burst. In this case, the lower lipophilicity of the acid does not justify the observed result and, therefore, other factors must be involved, probably the different genetic background and protein profile of both cell lines. In fact, P53-null CaCo-2 cells, cell line from which C2BBe1 cells are cloned, do not express endogenous ABCB1 transporter (known to be a multidrug efflux transporter),[33] in contrast with WiDr cells.[34] This may be related with the observed inhibitory effect of OHCA (**3**).

In relation to other structure–activity relationships (SAR), the presence of a catechol group, as in CAHA (**4**), seems to be the best structural feature for achieving effective antiproliferative activity in the studied colon cancer cells as well as in inhibition of human neutrophils' oxidative burst. The substitution of a hydroxyl group of the CAHA (**4**), by a methoxyl group, as in FAHA (**5**), led to a decrease in the antiproliferative activity, although for the higher concentrations, FAHA (**5**) was very effective in reducing cell proliferation. The addition of a supplementary hydroxyl group, as in OHCAHA (**6**), also led to a decrease in the anti-proliferative activity, particularly in C2BBe1 cells, although this decrease was lower than that caused by hydroxyl group substitution by a methoxyl group.

As amide CAHA (**4**) revealed to be the most cytotoxic compound, further studies were carried out in order to better understand the mechanisms of colon cancer cell death. Compound CAHA (**4**) revealed that its antiproliferative effect on C2BBe1 cells was related to a cell cycle blockade in G2/M phase. It is known that P53-null cancer cells lack G1 checkpoint, depending on checkpoint kinases for G2/M checkpoint.[35] Considering that C2BBe1 cells do not express P53 protein (data not shown), a possible DNA damage induced by CAHA (**4**) could retain cells in G2/M phase for DNA repair or apoptosis pathways activation. On the other hand, in WiDr cells CAHA (**4**) induced cell cycle arrest in S phase. The mutation of the *TP53* gene in WiDr cells is described by ATCC to prevent them from regulating P21 protein following DNA damage. P21 is a cyclin dependent kinase inhibitor responsible for the regulation of cell cycle G1 arrest. Generally, DNA damages induce an increase in P53 levels and a consequent P21 transcription, in order to activate G1 checkpoint and allow cells to repair damages or proceed to apoptosis.[36] In a previous study, CAHA (**4**) compound proved to increase P53 levels in breast cancer cells.[21] Thus, in WiDr cells, a possible mutant P53 increment may led to cell cycle arrest in S phase, since G1 checkpoint is not activated.

A cytotoxic effect induced by CAHA (**4**) was also observed in a dose-dependent manner, with a decrease on cell viability followed by an increase of early apoptotic cell population in both cell lines. This fact corroborates the presence of the apoptotic peak in WiDr cells. Moreover, the increment of aggregates/monomers ratio indicates a decrease of mitochondrial membrane potential, showing that CAHA (**4**) induces mitochondrial dysfunction. A reduction of mitochondrial membrane potential is an important event in the activation of apoptosis intrinsic pathway.[37] Interestingly, the cytotoxic effect was most evident in WiDr cells, allowing us to infer that P53 may play an important role in the apoptotic mechanism induced by CAHA (**4**).

Several studies demonstrated recently the anti-tumor effect of several phenolic compounds, like quercetin,[38] resveratrol,[39] curcumin[40] and caffeate derivatives.[13,17,21,41] Although several mechanisms underlying their anticancer effect have been proposed, a common denominator exists, their anti- and prooxidant activity.[21,42,43] Regarding hydroxycinnamic acid derivatives, it is of paramount importance taking into account that their antioxidant or prooxidant nature depends on the concentration used.[44]

In order to understand the anti-cancer mechanism of CAHA (**4**), intracellular production of $O_2^{\cdot-}$, peroxides and GSH was evaluated. Data obtained revealed an increase of intracellular production of peroxides that is accompanied by an increase of GSH production. GSH detoxifies any H_2O_2 formed by transferring the energy of the reactive peroxides to glutathione,[45] reason why significant differences in peroxides may not be seen. In turn, intracellular $O_2^{\cdot-}$ analysis after CAHA (**4**) treatment revealed an increment of its production in a dose-dependent manner, reaching almost 3-fold higher levels than control in WiDr cells. Given these results, it appears that $O_2^{\cdot-}$ could be the main ROS implicated in cell death mechanism of CAHA (**4**). The most pronounced production of $O_2^{\cdot-}$ observed in WiDr cells can be related with the presence of P53 protein, which is absent in C2BBe1 cells. Budanov et al. described the direct correlation between P53 overexpression and ROS accumulation in cancer cells in response to genotoxic stress.[46] $O_2^{\cdot-}$ is a by-product of mitochondrial respiratory chain, which can produce oxidative damage in mitochondria itself, but it can also diffuse away from its origin site and cause damages in many different macromolecules, like DNA, phospholipids and proteins.[45,47] Thus, the accumulation of $O_2^{\cdot-}$ induced by CAHA (**4**) could be an oxidative trigger for DNA damage and mitochondrial dysfunction, demonstrated by mitochondrial membrane potential decrease, leading cells to apoptosis.

5. Conclusion

In summary, new structure–activity relationships on hydroxycinnamic acid derivatives were achieved concerning inhibition of human neutrophils' oxidative burst and inhibition of colon cancer cells proliferation, showing that the presence of a catechol group is very important for both activities and that, generally, amides are more potent than the corresponding acids. A phenolic amide derivative CAHA (**4**) was found to be the most active compound in all the performed biological activities capable of inhibiting human neutrophils' oxidative burst, restraining the inflammatory process, and inhibiting growth of colon cancer cells, acting as antioxidant in conditions of excessive ROS and RNS production and prooxidant in the intracellular medium of colon cancer cells, triggering mitochondrial dysfunction that leads cells to apoptosis.

Please cite this article in press as: Tavares-da-Silva, E. J.; et al. *Bioorg. Med. Chem.* (2016), http://dx.doi.org/10.1016/j.bmc.2016.05.065

Altogether, these achievements can contribute to the understanding of the relationship between antioxidant and anticancer activities and based on the SAR established, can be the starting point to find more effective phenolic compounds as anticancer agents.

Acknowledgements

We would like to thank FCT (Fundação para a Ciência e Tecnologia, Portugal) for the financial support to IBILI-FMUC through PEst-C/SAU/UI3282/2013, to CNC.IBILI through strategic project UID/NEU/04539/2013 and to Applied Molecular Biology Unit, Institute of Immunology and University Clinic of Hematology, Faculty of Medicine of Coimbra, for the data acquisition of flow cytometry studies. UCIBIO, REQUIMTE also acknowledges European Union (FEDER funds POCI/01/0145/FEDER/007728) and National Funds [FCT/MEC (Ministério da Educação e Ciência)] under the Partnership Agreement PT2020 UID/MULTI/04378/2013.

Marisa Freitas and Ana Salomé Pires acknowledge FCT the financial support for the Pos-doc grant (SFRH/BPD/76909/2011) and for the PhD grant (SFRH/BD/75300/2010), respectively, in the ambit of 'QREN e POPH e Tipologia 4.1 e Formação Avançada', cosponsored by FSE and by national funds of MCTES.

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