JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Anticancer Activity of Olive Oil Hydroxytyrosyl Acetate in Human Adenocarcinoma Caco-2 Cells

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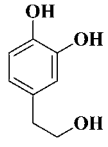
ABSTRACT: The anticancer activity of hydroxytyrosyl acetate (HTy-Ac) has been studied in human colon adenocarcinoma cells. Gene expression of proteins involved in cell cycle (p21, p53, cyclin B1, and cyclin G2) and programmed cell death (BNIP3, BNIP3L, PDCD4, and ATF3), as well as phase I and phase II detoxifying enzymes CYPA1 and UGT1A10, were evaluated by reverse transcription polymerase chain reaction after 24 h of exposure of Caco-2/TC7 cells to 5, 10, and 50 μM of HTy-Ac. The results show that HTy-Ac inhibited cell proliferation and arrested cell cycle by enhancing p21 and CCNG2 and lowering CCNB1 protein expression. HTy-Ac also affected the transcription of genes involved in apoptosis up-regulating of BNIP3, BNIP3L, PDCD4, and ATF3 and activating caspase-3. In addition, HTy-Ac also up-regulated xenobiotic metabolizing enzymes CYP1A1 and UGT1A10, thus enhancing carcinogen detoxification. In conclusion, these results highlight that HTy-Ac has the potential to modulate biomarkers involved in colon cancer.

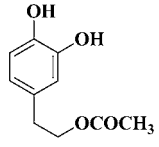
KEYWORDS: *Antiproliferative effect, Caco-2/TC7 cells, colon cancer, hydroxytyrosyl acetate, virgin olive oil phenols*

INTRODUCTION

There is evidence that virgin olive oil polyphenols induce beneficial effects on cardiovascular health.[1] In fact, The European Food Safety Authority (EFSA) has recently approved a health claim on the effectiveness of ingesting 5 mg/d of olive oil polyphenols on protecting LDL from oxidation.[2] Furthermore, regular consumption of olive oil has been related with a lower incidence of cancers, including colon cancer,[3] which has been linked to its content in polyphenols. In fact, treating human colon adenocarcinoma cells with olive oil phenols inhibits initiation, promotion, and metastasis of colon carcinogenesis processes.[4,5] In this sense, extra virgin olive oil is rich in a variety of phenolic compounds, mainly constituted by secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol, HTy) and of 2-(4-hydroxyphenyl)ethanol (tyrosol), and 2-(3,4-dihydroxyphenyl)ethyl acetate (hydroxytyrosyl acetate, HTy-Ac),[6] along with minor amounts of free HTy (Figure 1). Partial hydrolysis of secoiridoid derivatives and HTy-Ac during gastric and intestinal digestion has been widely described,[7,8] which increases HTy concentration at the colonic level. Therefore, extensive investigation has focused on HTy as a chronic disease preventive agent. In particular, anticancer properties of HTy have already been demonstrated in colonic cancer cell lines,[9−11] although the mechanisms involved remained to be studied. Recently, our research group has evaluated changes in gene expression in human adenocarcinoma cells (Caco-2 cells) treated with HTy by using transcriptome analysis which was later confirmed by reverse transcription polymerase chain reaction (RT-PCR) analysis. HTy has shown the capacity to alter genes related with cancer prevention in a dose-dependent manner by arresting the cell cycle, which could be associated with their capacity to modulate the transcription of specific genes involved in apoptosis. Moreover, HTy up-regulated xenobiotic metabolizing enzymes CYP1A1 and UGT1A10 (phase I and phase II, respectively), enhancing carcinogen detoxification.[12] These results highlight the potential of HTy to modulate molecular mechanisms involved in colon cancer. However, little attention has been paid to other phenolic compounds present in virgin olive oil, such as HTy-Ac (Figure 1).[6] The evaluation of the effects of HTy-Ac could help to complete the understanding of the overall chemopreventive effect of virgin olive oil in the colon. To date, HTy-Ac has shown protection effects against oxidative DNA damage in blood cells,[13] as well as against iron-induced oxidative stress in human cervical cells (HeLa).[14] Moreover, we have previously reported higher intestinal absorption of HTy-Ac in comparison with free HTy,[15] as well as the ability of HTy-Ac to protect human HepG2 cells against oxidative damage induced by t-BOOH, preventing radical formation and



Hydroxytyrosol (HTy)



Hydroxytyrosyl acetate (HTy-Ac)

Figure 1. Chemical structure of hydroxytyrosol (HTy) and hydroxytyrosyl acetate (HTy-Ac).

Received: December 3, 2012
Revised: March 1, 2013
Accepted: March 1, 2013
Published: March 1, 2013

modulating antioxidant defenses.[16] Bearing this in mind, the aim of the present study was to study the potential anticancer activity of HTy-Ac in human colon adenocarcinoma Caco-2/TC7 cells by analyzing its effects on cell cycle, apoptosis, and xenobiotic metabolism.

MATERIALS AND METHODS

Materials. Hydroxytyrosyl acetate (HTy-Ac) was synthesized from hydroxytyrosol (HTy) in ethyl acetate after incubation with *p*-toluenesulfonic acid and further purified by column chromatography, following a patented procedure.[17] HTy was recovered with 95% purity from olive oil wastewaters following a patented industrial system.[18] All reagents were purchased from Invitrogen (Paisley, UK) unless otherwise stated.

Cell Culture. The human Caucasian colon adenocarcinoma cell line Caco-2 (TC7 clonal cells) was kindly provided by Dr. Monique Rousset (INSERM, Paris, France). Caco-2/TC7 cells were grown in 75 cm^2 flasks and maintained in Dulbecco modified Eagle's medium (DMEM) (without phenol red) supplement with 20% fetal calf serum, 1% (v/v) nonessential amino acids, 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 μg/L streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. All cells used were between passages 50 and 60.

Preparation of Test Solutions. Standard stock solutions (50, 20, and 10 mM) of HTy-Ac were prepared in 10% DMSO with deionized water. Then, 10 and 100 times diluted solutions were prepared from stock solutions by adding distilled water, yielding a range of concentrations (5000, 2000, 1000, 500, 200, 100, 50, 20, and 10 μM, 1% DMSO) that were ultimately diluted with serum-free DMEM to prepare test solutions (500, 200, 100, 50, 20, 10, 5, 2, and 1 μM, 0.1% of DMSO). Concentrations were checked by HPLC to confirm complete dissolution in aqueous DMEM media.

Cell Proliferation Assay (BrdU). The effect of HTy-Ac on Caco-2 cell proliferation was evaluated using a chemiluminescent immunoassay ELISA kit (cat. no. 11 669 915 001, Roche Applied Science, Germany) based on the measurement of BrdU incorporation into genomic DNA during its synthesis in proliferating cells. Briefly, Caco-2/TC7 cells were seeded (10^4 cells per well) in black 96-well plates with clear bottoms and exposed to different concentrations of HTy-Ac (1, 2, 5, 10, 20, 50, 100, 200, and 500 μM) for 24 h in a humidified atmosphere (37 °C, 5% CO_2). Afterward, cells were labeled by adding BrdU for 4 h and subsequently anti-BrdU antibodies. The immune complexes were detected by the subsequent substrate (tetramethylbenzidine) reaction and quantified by measuring the luminescence using a scanning multiwall spectrophotometer in a microplate reader (BIO-TEK Instruments, Inc., Winooski, VT) after shaking the plate thoroughly for 1 min. Each phenol was tested six times. Results are expressed as the percentage of cell growth referred to untreated cells.

Quantification of Apoptosis by CaspACE. Apoptosis induced by the treatment of different concentration of HTy-Ac in Caco-2 cells was evaluated by the measurement of caspase-3 protease activity by using a colorimetric CaspACE Assay System kit (G7220, Promega), which uses a substrate labeled with the chromophore *p*-nitroaniline (pNA). Briefly, cells were grown in 6-well culture plates (10^4 cells per well) in a humidified atmosphere (37 °C, 5% CO_2). Cells were allowed to adhere to the plate surface for 36 h before being exposed to 2, 10, and 50 μM of HTy-Ac for 24 h. Each polyphenol concentration was tested three times. Then, 2 μL of DEVD-pNA substrate (10 mM stock solution) and 20 μM of caspase inhibitor Z-VAD-FMK were added to each well at the same time. Cleavage of the substrate by caspase-3 produced yellow pNA, which was measured spectrophoometrically at 410 nm. Untreated cells and cells treated with 5 μM of camptothecin, which induces apoptosis in Caco-2 cells under these conditions, were used as a negative and positive control, respectively.

Cell Cycle Analysis by Flow Cytometry. Caco-2 cells were seeded in 25 cm^2 flask (10^6 cells) and were allowed to adhere to the flask surface for 36 h before being exposed to different concentrations of HTy-Ac (2, 5, 10, 50, 100, and 200 μM, 0.1% DMSO final concentration) for 24 h. After phenol treatment, cells were washed twice with PBS and collected by trypsinization. Cells were suspended in DMEM supplement with 20% fetal calf serum (FBS), further centrifuged at 200*g* for 6 min at 4 °C, and resuspended in 0.5 mL of DMEM supplement with 20% FBS. Subsequently, cells were fixed in 3.5 mL of ice-cold ethanol/PBS (70:30) for 2 h. Fixed cells were centrifuged at 200*g* for 5 min and the pellet was resuspended in 4 mL of PBS and centrifuged again at the same conditions in order to completely remove ethanol solution. After removing the supernatant, cells were treated with 0.5 mL of 0.1% Triton X-100 in PBS containing DNase free RNase A (10 mg/mL) and 1 mg/mL propidium iodide (PI, Invitrogen Molecular Probes) for 30 min at 4 °C in the darkness, to stain nuclei cells. Samples were filtered through a 50 μm nylon mesh filter and analyzed by acquiring 20 000 events using a FACSCalibur flow cytometer (Becton Dickinson, BD) equipped with Cell QuestPro software (BD) for data acquisition and with FlowJo software (Tree Star, Ashland, OH) for data analysis. Data are presented as median values ± SD of three experiments.

RT-PCR. Changes in expression of 10 genes were verified by quantitative TaqMan Real Time PCR (Taqman System, Applied Biosystems) in independent experiments. Caco-2 cells were exposed to different concentrations of HTy-Ac (5, 10, and 50 μM, 0.1% DMSO) for 24 h in triplicate. Total RNA was isolated using a QIAGEN RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). RNA quality was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies), and RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer system (Nanodrop Technologies). Only samples with a ratio (Abs_{260}/Abs_{280}) between 1.8 and 2.1 were used for RT-PCR analysis.

Primers and probes from target genes were purchased from Applied Biosystems: cyclin B1 (CCNB1), Hs01030097_m1; cyclin G2 (CCNG2), Hs01548158_m1; BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), Hs00969289_m1; BCL2/adenovirus E1B 19 kDa interacting protein 3-like (BNIP3L), Hs00188949_m1; programmed cell death 4 (neoplastic transformation inhibitor) (PDCD4), Hs00913960_m1; UDP glucuronosyltransferase 1 family, polypeptide A10 (UGT1A10), Hs00166592_m1. The rest of selected genes, primers, and probes used for the cDNA synthesis were designed using ABI PRISM Primer Express Software v.2.0 (Applied Biosystems). Primers were purchased from Sigma-Genosys Ltd. (Haverhill, UK) and probes from Applied Biosystems. The probes were labeled with a 5′-reporter dye FAM (6-carboxyfluorescein) and 3′-quencher dye TAMRA (6-carboxytetramethylrhodamine). Primers were as follows: p21waf1/cip1, 5′-CTG GAG ACT CTC AGG GTC GAA-3′ (forward), 5′-CGG CGT TTG GAG TGG TAG A-3′ (reverse), and 5′-ACG GCG GCA GAC CAG CAT GAC-3 (probe); ATF3, 5′-GCG GAG CCT GGA GCA A-3′ (forward), 5′-AGC ACT CAC TTC CGA GGC A-3′ (reverse), and 5′-ATG CTT CAA CAC CCA GGC CAG GTC T-3′ (probe); CYP1A1 sense, 5′-CAA GGA CCT GAA TGA GAA GTT CTA CA-3′ (forward), 5′-GTC TGT GAT GTC CCG GAT GTG-3′ (reverse), and 5′-TTG TAG TGC TCT TGA CCA TCT TCT GCA TGA-3′ (probe); p53, 5′-TGC AAT AGG TGT GCG TCA GAA-3′ (forward), 5′-CCA GTG CAG GCC AAC TTG TT-3′, and 5′-ACC CAG GAC TTC CAT TTG CTT TGT CCC-3′ (probe).

Single-stranded cDNA was synthesized from 750 ng of total RNA in a 20 μL reaction volume using a qScript cDNA SuperMix kit (Quanta, Biosciences), which provides a sensitive and easy-to-use solution for two-step RT-PCR, and following thermal retrotranscription conditions according to the manufacturer's protocol. Then, RT-PCR was run in a microamp optical 96-well plate using a total reaction volume of 20 μL in a MicroAmp Optical Fast 96-well reaction plate covered by optical adhesive covers and using TaqMan Gene Expression RT-PCR Master Mix (Applied Biosystems). Twenty nanograms of total cDNA and prime and probe concentrations ranging from 100 to 500 nmol/L were used. Real-time RT-PCR conditions were as follows: AmpliTaq gold activation for 10 min at 95 °C, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Reactions were carried out in triplicate and data were analyzed by StepOne Software (v 2.0) using a standard curve to quantify the amount of mRNA obtained. Standard curves were produced for each

set of primes and probes using 5, 10, 20, 40, and 80 ng total RNA/reaction. Expression levels of target genes were normalized on the basis of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_ml) utilizing the standard curve method for quantification.

Statistical Analysis. Data were tested for homogeneity of variances using the test of Levene, prior to statistical analysis. Multiple comparisons were carried out using one-way ANOVA followed by Bonferroni tests when variances were homogeneous and Tamhane test when variances were not. The level of significance was established at $p < 0.05$. The statistical package SPSS (version 19.0) was used.

RESULTS

Antiproliferative Activity of HTy-Ac in Caco-2 Cells. The effects of HTy-Ac treatment for 24 h on Caco-2 cell proliferation were evaluated by measuring BrdU incorporation into genomic DNA during its synthesis in proliferating cells (Figure 2). HTy-Ac exerted a dose-dependent inhibitory effect on cell proliferation, inducing a statistically significant decrease for concentrations ≥5 μM.

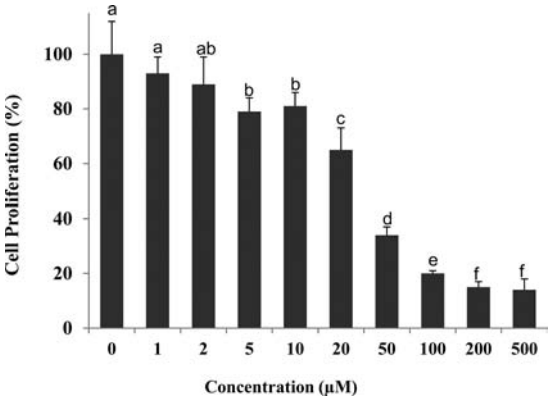


Figure 2. DNA synthesis in proliferating Caco-2 cells after 24 h of treatment with hydroxytyrosyl acetate (HTy-Ac) at different concentrations (1, 2, 5, 10, 20, 50, 100, 200, and 500 μM). Values are expressed as mean ± SD of six determinations. Different letters denote significant differences at $p < 0.05$.

Effects of HTy-Ac on Cell Cycle by Flow Cytometry. After Caco-2 cells were treated for 24 h with concentrations of HTy-Ac ranging from 2 to 200 μM, the percentage of cells in the different cell phases was analyzed using propidium iodide staining and flow cytometry (Figure 3). Untreated cells showed the following distribution after 24 h: 34.3% in G_0/G_1 phase, 41.4% in S phase, and 24.3% in G_2/M phase, whereas cells treated with HTy-Ac up to 10 μM showed an accumulation of cells in S phase, concomitant with a decrease in the proportion of cells in the G2/M phase. The highest concentrations assayed (100−200 μM) started blocking the cell cycle at G0/G1 and S phases with the consequent decrease of cells at G2/M phase.

Effect of HTy-Ac on Apoptosis Induction in Caco-2 Cells. In order to determine whether HTy-Ac treatment may affect apoptotic Caco-2 cell death, caspase-3 was evaluated in Caco-2 cells. Figure 4 shows that activation of caspase-3 takes place in a dose-dependent manner from 10 μM after a 24 h incubation period.

Effects on Gene Expression. Gene expression of proteins involved in cell cycle [CDKN1A (p21), TP53 (p53), CCNB1 and CCNG2] and programmed cell death (BNIP3, BNIP3L, PDCD4 and ATF3), as well as of the phase I and phase II detoxifying enzymes CYPA1 and UGT1A10, respectively, were

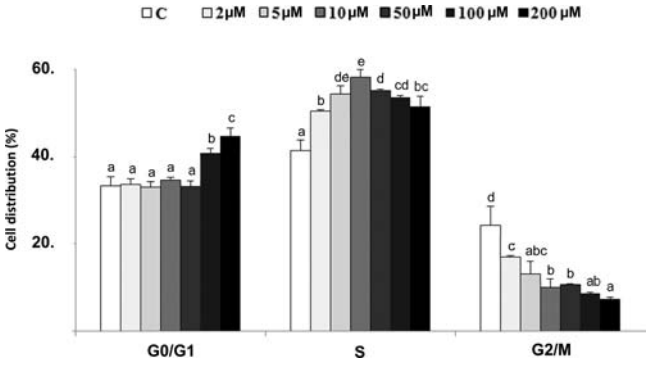


Figure 3. Changes in Caco-2cell cycle distribution when exposed to different concentrations of hydroxytyrosyl acetate (HTy-Ac) after 24 h of incubation. The cell percentage in each cycle phase was determined by flow cytometry analysis after incubating the cells with RNase (5 mg) and PI (0.5 mg) at 37 °C for 30 min. Data are expressed as mean values ± SD ($n = 3$). Different letters denote significant differences between control and treated cells of each phase, $p < 0.05$.

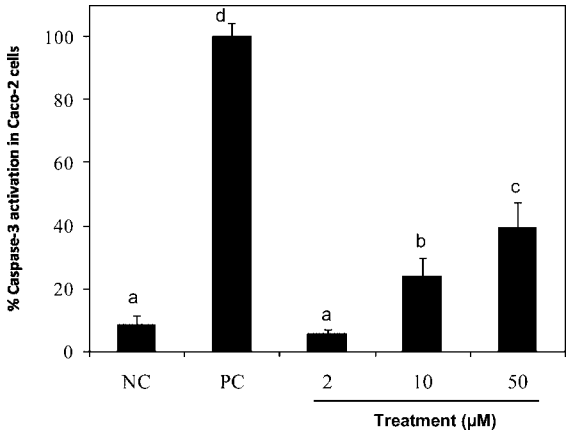


Figure 4. Caspase-3 activation in Caco-2 cells after 24 h of treatment with hydroxytyrosyl acetate (HTy-Ac) at 2, 10, and 50 μM. Values are expressed as mean ± SD from three independent experiments. NC is the negative control (untreated cells) and PC is the positive control [cells with apoptosis induced by camptothecin (5 mM)]. Different letters denote significant differences at $p < 0.05$.

evaluated using RT-PCR after exposure of Caco-2 cells to 5, 10, and 50 μM of HTy-Ac for 24 h. The selection of these genes was based on a previous study carried out in our group,[12] where the anticancer effects of hydroxytyrosol compared with its lipophilic derivative, hydroxytyrosyl ethyl ether, in human adenocarcinoma cells (Caco-2 cells) were evaluated using transcriptome analysis. In Table 1 are depicted the expression levels of the selected genes. Results show that HTy-Ac affected Caco-2 cell proliferation by enhancing p21 and CCNG2 gene expression and lowering CCNB1 protein expression in a dose-dependent manner ($p < 0.001$), whereas no change took place in p53 in comparison with untreated cells. This outcome demonstrates that HTy-Ac has the ability to arrest cell cycle and reinforces the results obtained regarding the analysis of cell cycle and BrdU incorporation.

Alternatively, genes associated with proapoptotic functions, such as BNIP3 and BNIP3L, were up-regulated after treatment with HTy-Ac from the lowest concentration tested, 5 μM, as well as genes associated with tumor suppression, such as PDCD4 and ATF3. Particularly, ATF3 showed a 4-fold change in its expression at the highest concentration assayed. The

Table 1. Expression Levels of the Selected Genes Quantified Using RT-PCR Analysis In Caco-2 Cells after Exposure to Hydroxytyrosyl Acetate (HTy-Ac) at Different Concentrations (5, 10, and 50 μM) [a]

gene name[b]	gene symbol	RT-PCR (fold change)[c]			
		0 μM	5 μM	10 μM	50 μM
Cell Cycle					
cyclin-dependent kinase inhibitor 1A (p21, Cip 1)	CDKN1A	1.00 ± 0.10 a	1.30 ± 0.10 b	1.40 ± 0.10 b	3.70 ± 0.40 c
tumor protein p53	TP53 (p53)	1.02 ± 0.07 a	1.20 ± 0.30 a	1.20 ± 0.10 a	1.40 ± 0.40 a
cyclin G2	CCNG2	0.99 ± 0.06 a	1.20 ± 0.10 b	1.70 ± 0.20 c	3.10 ± 0.20 d
cyclin B1	CCNB1	0.97 ± 0.03 a	0.65 ± 0.08 b	0.70 ± 0.10 b	0.54 ± 0.08 c
Programmed Cell Death					
BCL2/adenovirus E1B 19 kDa interacting protein 3	BNIP3	0.99 ± 0.06 a	1.40 ± 0.10 b	1.40 ± 0.10 b	1.90 ± 0.20 c
BCL2/adenovirus E1B 19 kDa interacting protein 3-like	BNIP3L	0.99 ± 0.06 a	1.30 ± 0.20 b	1.50 ± 0.10 b	1.90 ± 0.20 c
programmed cell death 4	PDCD4	1.02 ± 0.05 a	1.20 ± 0.10 b	1.20 ± 0.10 b	1.50 ± 0.10 c
activating transcriptor factor 3	ATF3	0.97 ± 0.06 a	1.10 ± 0.10 a	1.50 ± 0.10 b	4.20 ± 0.40 c
Phase I and Phase II Enzymes					
UDP glucuronosyltransferase 1 family, polypeptide A10	UGT1A10	1.00 ± 0.10 a	1.40 ± 0.20 b	2.20 ± 0.40 c	2.50 ± 0.70 c
cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	0.90 ± 0.05 a	1.00 ± 0.10 a	2.20 ± 0.10 b	8.50 ± 0.90 c

[a]Results are expressed as fold change determined as the ratio calculated by real-time RT-PCR between Caco-2 cells treated with HTy-Ac and cells treated with equal amounts of vehicle DMSO. [b]These genes were differentially expressed in Caco-2 cells in response to HTy-Ac exposure. For all genes identified as differentially expressed a p-value was calculated using a t-test ($p < 0.05$). [c]Values <0 indicate down-regulation, values >0 indicate up-regulation.

alteration of these genes, which are involved in the regulation of tumor suppressors, along with the activation of caspase-3, could be the mechanism underlying the observed inhibition of cell proliferation.

Finally, HTy-Ac treatment up-regulated genes involved in drug metabolism and detoxification processes in Caco-2 cells, leading to significant differences in the level of expression of both UGT1A10 and CYP1A1 from 5 and 10 μM, respectively. In summary, these results highlight the potential of HTy-Ac to modulate molecular mechanisms involved in colon cancer.

DISCUSSION

Extra virgin olive oil is rich in phenolic compounds that have been associated with a reduced incidence and prevalence of cancer, including colorectal cancer.[3] Although the olive oil phenolic fraction is a complex mixture of HTy derivatives, in vitro studies have been approached using free HTy taking into account the partial hydrolysis of secoiridoid derivatives and HTy-Ac.[7,8] Recently, Pinto et al[7] investigated the absorption and metabolism of secoiridoids in isolated, perfused segments of the jejunum and ileum, in addition to Caco-2 cells identifying unmetabolized compounds along with reduced and glucuronidated forms of secoiridoid derivatives and free HTy. In line with these results, HTy-Ac showed high stability under gastric conditions,[8] being significantly better absorbed than free HTy in Caco-2 cells[15] identifying nonmetabolized HTy-Ac (37.5%) and its glucuronide derivative (17.4%), together with free HTy (38.4%) and homovanillyl alcohol (6.7%). These results encouraged us to extend our understanding of the biological activity of HTy-Ac in human adenocarcinoma Caco-2 cells.

In Mediterranean countries typical daily consumption of 30 mL of virgin olive oil with a concentration ranging between 180 and 300 mg/kg of olive oil phenols would result in an estimated intake of about 5.4−9.0 mg of olive oil phenols per day, which meets the recommended intake of 5 mg/d of olive oil polyphenols.[2] A recent study has shown that a single ingestion of 40 mL of olive oil with high (366 mg/kg) and medium (164 mg/kg) contents of polyphenolic compounds leads to hydroxytyrosol metabolites concentration in plasma of 15−30 μM.[19] In addition, in a human ileostomy study it was shown that up to 66% of the ingested olive oil phenols were absorbed from the small intestine.[20] Taking into account these premises, up to 50 μM of phenolic compounds should reach the gut in order to detect the highest concentration of 30 μM in plasma described by Covas et al.[19] On the basis of these results, 5, 10, and 50 μM were selected, as they are realistic physiological concentrations.

The results observed in the present work confirm the capacity of HTy-Ac to inhibit Caco-2 cell proliferation and to arrest cell cycle progression by blocking S phase at the lower concentrations studied (from 2 to 50 μM) and G0/G1 phase at the higher (100 and 200 μM).

This outcome is partially in agreement with its precursor HTy,[12] although higher HTy concentrations (between 50 and 200 μM) were required to arrest the cell cycle at the S phase. In this same study, HTy biological activity was compared with that of its lipophilic synthetic derivative, hydroxytyrosyl ethyl ether. The latter phenol compound arrested cell cycle at the S phase, although lower concentrations ranging from 50 to 200 μM blocked cell cycle at G0/G1, in accordance with that observed for HTy-Ac. Therefore, the blocking of the hydroxyl group of HTy phenyl moiety to generate the lipophilic ester HTy-Ac and ether derivatives hydroxytyrosyl ethyl ether produced similar effects on cell cycle arresting and slightly higher and different effects than that shown by HTy, as described with other phenolic compounds with different chemical structures, such as ferulic and p-coumaric acids,[21] in the same cellular line. Similarly, Bernini et al.[22] observed a higher cell growth inhibitory activity for a novel ester of HTy with α-lipoic acid than with the natural parent compound HTy, arresting the cell cycle at the G2/M phase in HT-29 cells. The antiproliferative and cell cycle arresting effects previously described may be associated with the changes in gene expression of proteins involved in cell cycle after 24 h of exposure of HTy-Ac. The up-regulation of p21, involved in cell cycle arrest at G1, S or G2 phases, took place through a separate pathway than that of p53, since p53 expression remained unchanged. Furthermore, the capacity of HTy-Ac to arrest the cell cycle was confirmed by the induction of cyclin G2 and the reduction of cyclin B1, which

control the G1/S and G2/M phases, respectively. These results agree with the capacity of HTy to interfere in the initiation or progression of gastrointestinal cancer, although HTy-Ac was slightly more active.[12]

The antiproliferative effects of HTy-Ac could be associated with proapoptotic mechanisms, such as increasing caspase-3 activity and up-regulating genes such as BNIP3, BNIP3L, PDCD4, and ATF3. In particular, BNIP3 and BNIP3L are mitochondrial proteins that induce apoptosis when transitorily overexpressed. Furthermore, it has been described that BNIP3 is up-regulated in cells when fatty acid synthase (FASN) expression is repressed.[23] This relationship has been confirmed recently in an in vivo study carried out in mice, where the administration of metformin, an antidiabetic drug, blocked the enhancing effect of a high-energy diet on colon carcinoma growth together with reduced expression of FASN and up-regulation of BNIP3.[24] In line with these results, the olive oil polyphenol HTy inhibited FASN expression in human colorectal cancer SW620 cells,[11] although these authors did not evaluate the expression of BNIP3. Among the other genes selected to evaluate the involvement of HTy-Ac in apopototic process of Caco-2 cells, HTy-Ac also up-regulated the expression of PDCD4, in accordance with resveratrol, which up-regulated this novel tumor suppressor gene in SW480 colon cancer cells.[25] Finally, ATF3 is an interesting biomarker since its up-expression induces antitumor effects by accelerating the apoptotic rate. Accordingly, others flavonoids such as genistein and daidzein have also shown capacity to up-regulate ATF3 in colorectal HCT 116 cells.[26] In previous experiments, our group has evaluated the ability of HTy to induce apoptosis in Caco-2 cells.[12] HTy also showed the capacity to up-express BNIP3, BNIP3L, PDCD4, and ATF3 genes in Caco-2 cells. When HTy and HTy-Ac are comparatively studied, HTy-Ac is slightly more active, especially due to increasing ATF3 expression, doubling its expression level at 50 μM and inducing caspase-3 activation, requiring lower concentrations (10 μM for HTy-Ac versus 50 μM for HTy), to induce a statistical effect in its activity.

Finally, genes CYP1A1 and UGT1A10, which are also involved in the xenobiotic metabolism, were selected to better understand the anticancer activity of HTy-Ac. Polyphenols treatment with ellagic acid and urolithins have demonstrated their ability to alter the expression of these enzymes in Caco-2 cells.[27] In agreement, HTy-Ac treatment activated the expression of the genes encoding these enzymes in Caco-2 cells. In comparison with its precursor HTy, HTy-Ac showed a 2-fold effect in the expression at 10 and 50 μM.[12]

Altogether, HTy-Ac and its precursor HTy showed similar activity. HTy-Ac achieved the same effects as HTy at lower concentrations. Similar outcome was observed with hydroxytyrosyl ethyl ether and its precursor HTy.[12] The higher biological activity found for both lipophilic compounds HTy-Ac and hydroxytyrosyl ethyl ether, compared to HTy, may be related to their better absorption across the Caco-2 cell monolayer, previously described by Pereira-Caro et al.[28] and Mateos et al.[15] In agreement, Fabiani et al.[29] evaluated the chemopreventive potential of different olive oil extracts in HL60 cells and described a better correlation between secoiridoid derivatives (lipophilic precursor of HTy) than free phenyl alcohols (HTy and tyrosol) and lignans with antiproliferative and pro-apoptotic activity.

This study confirms the chemopreventive activity of a component of natural virgin olive oil, HTy-Ac, in Caco-2 cells by inducing cell cycle arrest, promoting apoptosis, and enhancing xenobiotic metabolism. These data shed light on the bioactivity of olive oil polyphenols, demonstrating their potential in the formulation of functional food or food supplements aimed at preventing the development of colonic cancer.

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Author Contributions

‖R.M. and G.P.-C. have contributed equally to this work.

Funding

This work was supported by projects AGL2007-66373-C04/ALI and CSD2007-00063 from the Consolider-Ingenio Program of the Spanish Ministry of Science and Innovation (CICYT), Grant (RTA2007-000036-00-00) from the Research National Institute of Food, Agriculture and Technology (INIA), Contract 110105090014 CSIC-IFAPA, and by funding from the Biotechnology and Biological Sciences and Council (UK). G.P.-C. is a predoctoral fellow of the National Research Institute of Food, Agriculture and Technology (INIA).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

HTy-Ac was kindly provided by José Luis Espartero from Seville University (Spain).

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