European Journal of Medicinal Chemistry 46 (2011) 439–446

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Preliminary communication

Synthesis of a novel ester of hydroxytyrosol and α-lipoic acid exhibiting an antiproliferative effect on human colon cancer HT-29 cells

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ARTICLE INFO

Article history:
Received 12 July 2010
Received in revised form
18 October 2010
Accepted 19 October 2010
Available online 30 October 2010

Keywords:
Hydroxytyrosol
α-Lipoic acid
Antiproliferative activity
Human colon cancer HT-29 cell line
2-Iodoxybenzoic acid (IBX)

ABSTRACT

A novel ester of hydroxytyrosol and α-lipoic acid was synthesized in satisfactory yield by original and simple procedures and evaluated about its antiproliferative activity on the human colorectal adenocarcinoma HT-29 cell line. The compound exhibited a cell growth inhibitory activity significantly more potent than the corresponding parent natural compounds, very likely due to the induction of cell cycle arrest in the G2/M phase. These data suggest that the novel ester might exert a more effective antitumour activity than hydroxytyrosol and α-lipoic acid.

1. Introduction

Bioactive compounds are extranutritional constituents that typically occur in foods. Epidemiological and prospective studies have reported several beneficial effects of these compounds on human health, particularly the protective activity from chronic-degenerative diseases such as cardiovascular and cancer diseases [1]. Among them, phenolic compounds, present in fruits and vegetables, show antioxidant and antiproliferative properties [2]. Due to the increasing interest in these products, particular attention has recently been paid to agro-industrial wastes as a source of antioxidants [3].

2-(3,4-Dihydroxyphenyl)ethanol (hydroxytyrosol, Fig. 1) is the most important component of the phenolic compounds found in virgin olive oil [4] and in olive oil wastewaters [5]. Furthermore, it has been identified and quantified in several Italian white and red wines [6]. It has recently attracted a lot of interest in view of its interesting pharmacological effects. For example, it exhibits antioxidant activity attributed to the *ortho*-diphenolic moiety [7], protects human erythrocytes against oxidative damage and low-density lipoprotein oxidation [8], induces cytochrome c-dependent apoptosis [9], prevents platelet aggregation, reduces the risk of cardiovascular [10] and neurodegenerative diseases [11], and exhibits antitumour activity on certain types of cancer [12–19]. It has recently been shown that hydroxytyrosol exerts growth inhibitory activity on human promyelocytic leukemia HL-60 cells [12–14], as well as human breast cancer MCF-7 [15,16] and colon cancer HT-29 cells [12,17–19].

Another important food component is a non-phenolic compound, 1,2-dithiolane-3-pentanoic acid also known as 6,8-thioctic acid or α-lipoic acid (Fig. 1) present mainly in wheat [20], potatoes [21], and red meat [22]. It is a naturally occurring cofactor of many multienzyme complexes found in prokaryotic and eukaryotic microorganisms, where it plays a pivotal role in energy metabolism [23]. During the last decade, α-lipoic acid has attracted increasing interest pharmacologically for its beneficial effect in the therapy of many diseases, including diabetes and diabetic neuropathy [24], atherosclerosis [25], Alzheimer's disease [26], and acquired immune deficiency syndrome (AIDS) [27]. Its well known antioxidant activity, related to the presence of the thiolic group [28], has also been shown to protect against tumourigenesis and cancer progression [29].

On the basis of the data in the literature concerning the biological and pharmacological properties of both hydroxytyrosol and α-lipoic acid, our preliminary studies were focused on providing molecular combinations obtained by joining the two biologically

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doi:10.1016/j.ejmech.2010.10.028

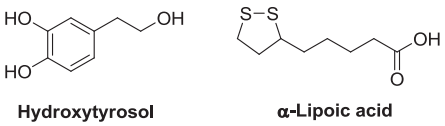


Fig. 1. The chemical structure of two bioactive compounds present in foods.

active molecules to produce one novel compound. A single compound exhibiting both the functionalities responsible for the biological activity of the parent compounds and a very likely enhanced lipophilicity should result potentiated in its biological activity. For this purpose, we projected the synthesis of the ester depicted in Fig. 2. This compound was evaluated for its effect on the proliferation of the human colorectal adenocarcinoma HT-29 cell line, compared to hydroxytyrosol and α-lipoic acid. The examination of digestive system tumour cells is of particular interest because bioactive compounds are expected to come into direct contact with gastrointestinal tumour cells at higher concentrations and the effect of metabolism is minimal.

2. Results and discussion

2.1. Chemistry

Commercially available 2-(4-hydroxyphenyl)-ethanol **1** (tyrosol) and α-lipoic acid **2** were used as starting materials for the preparation of the novel ester. Like hydroxytyrosol, tyrosol is a phenolic compound present in olive oil [4] and in olive oil wastewaters [5], but the presence of the phenolic group instead of the catecholic moiety substantially contributes to its low biological activities. Therefore, it is a compound of lesser value compared to hydroxytyrosol. However, as recently reported by us [30], it can be

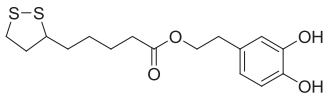
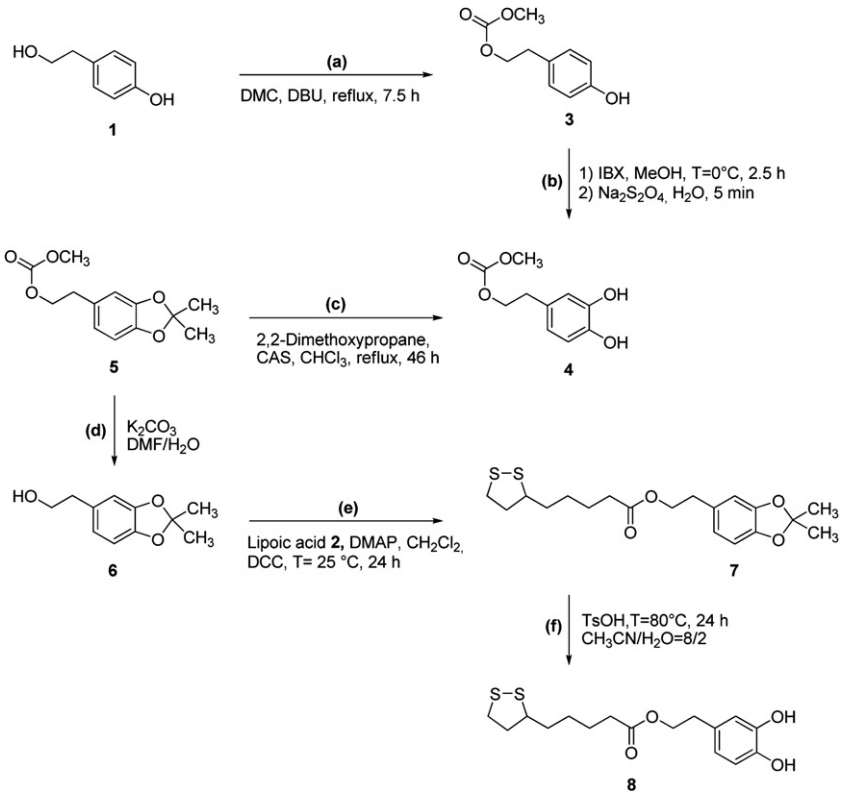


Fig. 2. The projected novel ester.

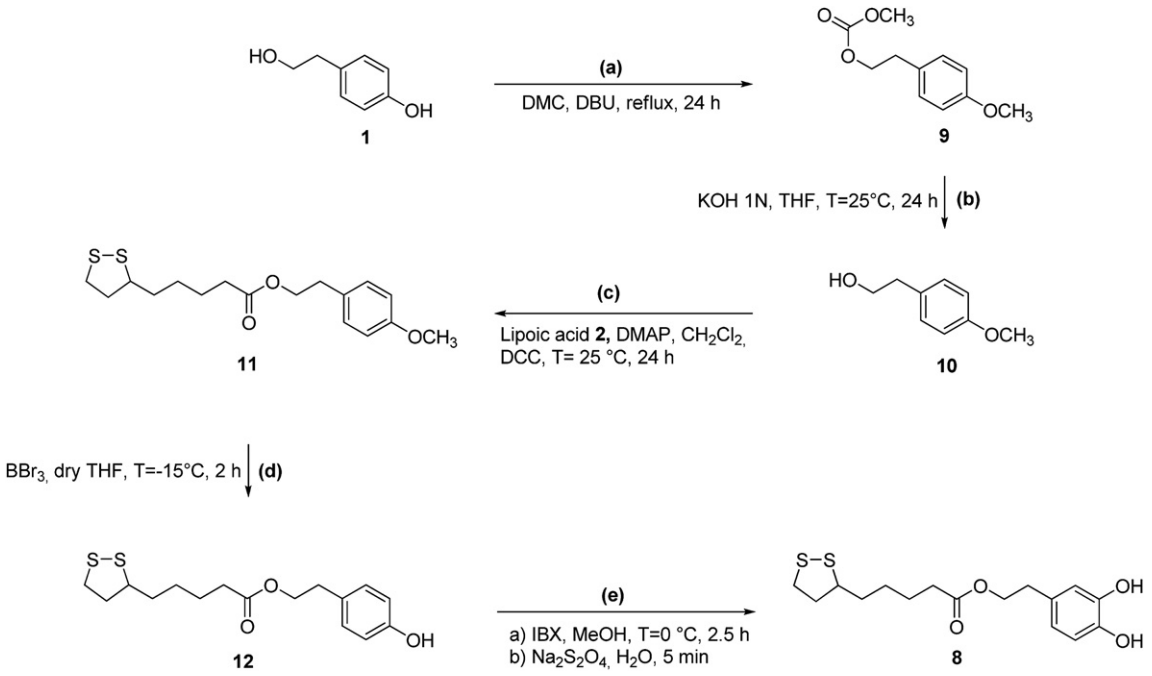
'valorised', representing a naturally occurring starting material for the preparation of the precious parent compound.

Considering the novelty of the ester, it was necessary to establish the appropriate reaction conditions for its synthesis. After several efforts, two routes were selected. In both, key steps were an esterification reaction between α-lipoic acid and tyrosol/hydroxytyrosol derivatives adequately protected on the phenolic moiety and a regioselective aromatic hydroxylation of the monohydroxylated precursor. The reagent of choice was 1-hydroxy-1-oxo-1H-1λ^5-benz[d][1,2]iodoxol-3-one (2-iodoxybenzoic acid, IBX) in combination with sodium dithionite ($Na_2S_2O_4$) [31]. This oxidative/reductive system is able to insert a hydroxyl group in the *ortho*-position to a phenolic group with a chemo- and a regio-selectivity similar to that naturally occurring in cytochrome P450 monooxygenases [32].

The first procedure to obtain the novel ester is described in Scheme 1. Tyrosol **1** was treated with dimethyl carbonate (DMC), an ecofriendly chemical used both as solvent and carboxymethylating reagent, in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base [33]. The corresponding product **3** was isolated in quantitative yield after about 7.5 h [Step (a)]. Following the aromatic hydroxylation of compound **3** with IBX and *in situ* reduction with $Na_2S_2O_4$, catecholic derivative **4** was isolated in very good yield (80%) and with a high degree of purity [Step (b)]. To improve the stability of this compound and eliminate the possible competitive reactivity of the phenolic groups in the esterification reaction, the catecholic moiety was protected as acetonide by using 2,2-dimethoxypropane in the presence of camphorsulphonic acid



Scheme 1. First synthetic route for the preparation of the novel ester.



Scheme 2. Alternative synthetic route for the preparation of the ester **8**.

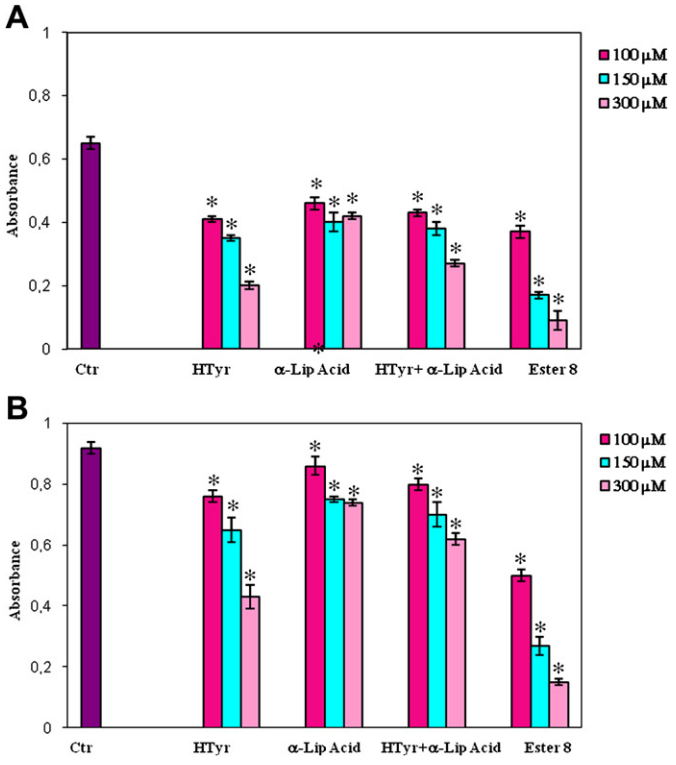


Fig. 3. Inhibition of HT-29 cell proliferation. HT-29 cells were treated with hydroxytyrosol (HTyr), α-lipoic acid, hydroxytyrosol (HTyr) plus α-lipoic acid, and the novel ester **8** at three different concentrations (100, 150 and 300 μM) for 24 h (Panel A) and 48 h (Panel B). Cell proliferation was assessed using the 5-bromo-2′-deoxyuridine (BrdU)-enzyme-linked immunosorbent assay (ELISA) and results were expressed as the mean ± S.D. of triplicates; $^{*}P < 0.05$; P values were calculated comparing the experimental results to control results. One representative experiment out of five independent experiments is illustrated.

(CAS) as catalyst, obtaining compound **5** [yield: 90%, Step (c)] [34]. After the selective and quantitative deprotection of the carbonate moiety under basic conditions [Step (d)], compound **6** was esterified with α-lipoic acid **2** under Steglich conditions, i.e. in the presence of 4-(dimethylamino)-pyridine (DMAP) and dicyclohexylcarbodiimide (DCC) in dichloromethane as solvent [35]. According to the literature [36], 0.5% of L-cysteine as polymerization inhibitor was added to the esterification mixture in order to prevent the well known polymerization of α-lipoic acid [37]. The corresponding ester **7** was isolated in high yield [90%, Step (e)]. Finally, the deprotection of the acetonide moiety produced the expected ester **8** in 62% yield [Step (f)]. With this route, the overall yield of the ester **8** was 40%.

An alternative and more efficient synthetic procedure is depicted in Scheme 2. First, we completely protected both the alcoholic and the phenolic group of tyrosol **1** with DMC/DBU. Extending the reaction time to 24 h, product **9** was isolated in quantitative yield [Step (a)]. The selective deprotection of the carbonate moiety under basic conditions in the presence of KOH quantitatively produced compound **10** [Step (b)]. The following esterification reaction of tyrosol derivative **10** with α-lipoic acid **2** under Steglich conditions and in the presence of L-cysteine afforded the ester **11** in 92% yield [Step (c)]. Finally, treatment of **11** with boron tribromide [38] produced the expected demethylated ester **12** in satisfactory yield [84%, Step (d)]. After the following aromatic hydroxylation with IBX/$Na_2S_2O_4$, the ester **8** was isolated in 80% yield. Using this procedure, the overall yield of the desired ester **8** was 62%, higher than the previously described synthesis and with five steps instead of six.

2.2. Antiproliferative activity on the human colorectal adenocarcinoma HT-29 cell line

The novel ester **8** was evaluated for its effect on the proliferation of the human colorectal adenocarcinoma HT-29 cell line compared to the parent natural compounds hydroxytyrosol and α-lipoic acid, either singularly or in combination. For this purpose, HT-29 cells were incubated with different solutions of compounds at concentrations of 100, 150, and 300 μM for 24 and 48 h. Cell proliferation

Table 1
Percentage of HT29 cell growth inhibition induced by cell treatment with hydroxytyrosol, α-lipoic acid, hydroxytyrosol plus α-lipoic acid, and the novel ester **8** for 24 h and 48 h.

Concentration (μM)	Time (h)	Hydroxytyrosol	α-Lipoic acid	Hydroxytyrosol + α-Lipoic acid	Ester **8**
100	24	20.7 ± 2.1	17.8 ± 5.0	23.6 ± 1.1	33.9 ± 2.7*
150	24	37.5 ± 3.7	25.5 ± 2.3	32.1 ± 1.4	69.6 ± 3.3*
300	24	64.2 ± 4.2	24.0 ± 4.6	50.5 ± 1.0	83.9 ± 4.2*
100	48	17.3 ± 2.5	6.5 ± 6.0	12.5 ± 2.1	45.6 ± 2.1*
150	48	29.3 ± 2.6	18.4 ± 3.2	23.8 ± 1.1	70.6 ± 4.8*
300	48	53.2 ± 4.1	19.5 ± 4.1	40.1 ± 2.3	83.6 ± 3.9*

Results were expressed as the mean ± S.D. of triplicates. *$P < 0.05$; P values were calculated comparing ester **8** results to results obtained with hydroxytyrosol and α-lipoic acid either singularly or in combination.

was assessed using the 5-bromo-2′-deoxyuridine (BrdU)-enzyme-linked immunosorbent assay (ELISA). The concentrations of compounds used in our experiments were found to have no effect on cell viability, as assessed by the viability assay (data not shown). As shown in Fig. 3 and Table 1, all the compounds, either singularly or in combination, were able to induce a significant dose-dependent inhibition of tumour cell growth after 24 h (Panel A) and 48 h (Panel B). However, the levels of growth inhibitory activity differed among the different compounds at all the dose-levels employed (Fig. 3, Table 1). Notably, the novel ester **8** induced a significant more potent cell growth inhibition at all the dose-levels compared to α-lipoic acid and hydroxytyrosol either singularly or in combination, reaching 83.9% of growth inhibitory activity at a concentration of 300 μM for 24 h (Table 1). The same compound **8** at a concentration of 150 μM for 24 h induced a tumour cell growth inhibition of 69.6% (Table 1). A comparable inhibitory activity was obtained using 300 μM of hydroxytyrosol for 24 h (Table 1), whereas this level of inhibitory activity could not be achieved by any of the dose-levels employed for either α-lipoic acid singularly or in combination with hydroxytyrosol at 24 or 48 h (Table 1). This trend was most evident from the data of the 48 h incubation, where 100 μM of the ester **8** induced 45.6% of growth inhibition (Table 1); a comparable value was obtained using either hydroxytyrosol singularly or in combination with α-lipoic acid at a concentration of 300 μM (Table 1). We also observed that the percentages of cell growth inhibition did not change in a time-dependent manner when 150 and 300 μM of the compounds were used, indicating that with these higher doses, the inhibitory activity had reached a plateau at 24 h. Our results, obtained by treatment of tumor cells with hydroxytyrosol or α-lipoic acid, are in agreement with previous data in the literature reporting that both hydroxytyrosol and α-lipoic acid exert an antitumour activity inducing growth inhibition of tumour cells [12–16,20]. In particular, it has been shown that hydroxytyrosol inhibits proliferation of leukemia HL-60 cells [12–14], as well as breast MCF-7 [15,16] and colon HT-29 [18] cancer cells. Overall, our data show that the novel ester **8** exhibits an antiproliferative effect on the human colorectal adenocarcinoma HT-29 cell line which is significantly more potent than that exerted by hydroxytyrosol and α-lipoic acid either singularly or in combination, suggesting that the novel compound might exert a more effective antitumour activity than its parent natural compounds.

To analyze the possible mechanism underlying the antiproliferative effect exerted by the novel ester **8**, HT-29 cell cycle progression was assessed following treatment with 100, 150, and 300 μM of ester **8** for 24 h. Hydroxytyrosol and α-lipoic acid, either singularly or in combination, were used as controls for the novel ester **8**. The DNA cell cycle distribution was quantified by flow cytometry. As shown in Fig. 4 and Table 2, all the compounds, either singularly or in combination, induced a significant cell cycle block in the G2/M phase, manifested by an increase of the cell number in the G2/M phase with a concomitant reduction in the number of cells in G0/G1 phase (Fig. 4, Table 2). This result is in agreement with previous studies, which have shown that hydroxytyrosol induces inhibition of the proliferation of human colon carcinoma cells via its ability to induce a cell cycle block in the G2/M phase [18]. Interestingly, accordingly to the cell growth inhibitory property, the novel ester **8** was capable of inducing a significant stronger block of the tumor cell cycle in G2/M phase compared to hydroxytyrosol and α-lipoic acid, either singularly or in combination (Fig. 4, Table 2). This result indicates that the antiproliferative effect exhibited by the novel ester **8** on the human colorectal adenocarcinoma HT-29 cell line is due to a cell cycle arrest in the G2/M phase.

3. Conclusions

A novel ester obtained by joining hydroxytyrosol with commercial α-lipoic acid was prepared in satisfactory yield and high purity and evaluated for its effect on the proliferation of the human colorectal adenocarcinoma HT-29 cell line. The results obtained showed that the ester exhibited an antiproliferative effect significantly more potent than the corresponding parent compounds, either singularly or in combination. Moreover, it induced a significantly stronger block of the cell cycle at the G2/M phase than hydroxytyrosol and α-lipoic acid, either singularly or in combination. This result indicates that the inhibition of cancer cell growth was mediated by the induction of a G2/M phase cell cycle arrest. The data suggest that this novel compound might exert a more effective antitumour activity than the parent natural compounds.

Further syntheses are currently in progress in our laboratories for the preparation of analogue derivatives which will be compared with the ester **8** and the corresponding parent compounds regarding their stability as well as antioxidant and antiproliferative activities on tumour cell lines.

4. Experimental

4.1. Chemistry

Materials and equipment: Solvents, α-lipoic acid, and reagents used were of analytical grade and were purchased from Aldrich Chemical Company (Milan, Italy). Hydroxytyrosol was synthesized following our previously published method [30d]; IBX was prepared as described in the literature [39]. Silica gel 60 F254 plates and silica gel 60 were supplied by Merck (Milan, Italy). ^{1}H NMR and ^{13}C NMR were recorded on a Bruker 200 MHz spectrometer using $CDCl_3$ (99.8% in deuterium), CD_3OD (99.8% in deuterium) or CD_3COCD_3 (99.8% in deuterium) as the solvent. All chemical shifts are expressed in parts per million (δ scale) and coupling constants in Hertz (Hz). HR-MS was recorded with a Micromass Q-TOF micro mass spectrometer (Waters).

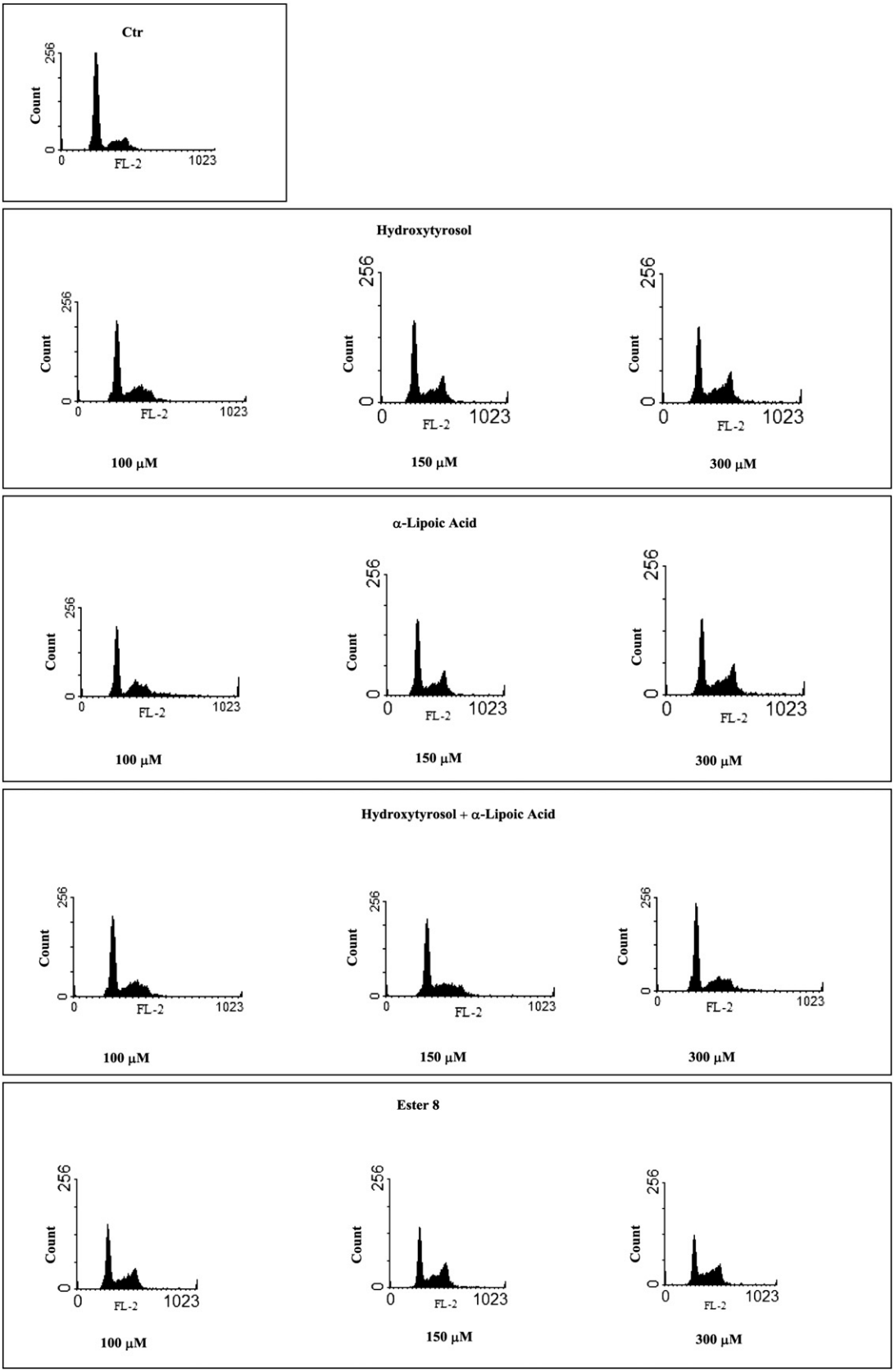


Fig. 4. Block of HT-29 cell cycle at the G2/M phase. HT-29 cells were treated with hydroxytyrosol, α-lipoic acid, hydroxytyrosol plus α-lipoic acid, and the novel ester **8** at three different concentrations (100, 150, and 300 μM) for 24 h. DNA cell cycle distribution was quantified by flow cytometry. One representative experiment out of five independent experiments is illustrated.

Table 2
Percentage of G0/G1, S and G2/M HT-29 cell cycle phase distribution. HT-29 cells were treated with hydroxytyrosol, α-lipoic acid, hydroxytyrosol plus α-lipoic acid, and the novel ester **8** for 24 h.

Treatment	Concentration (μM)	G0/G1 (%)	S (%)	G2/M (%)
Ctr	–	61.4 ± 1.1	21.0 ± 0.9	17.6 ± 1.0
Hydroxytyrosol	100	40.5 ± 1.3	22.5 ± 1.5	35.0 ± 1.0*
	150	40.3 ± 1.1	20.3 ± 1.4	36.7 ± 1.2*
	300	34.4 ± 0.8	20.1 ± 1.5	43.5 ± 2.1*
α-Lipoic Acid	100	43.5 ± 0.6	19.2 ± 1.5	36.9 ± 1.3*
	150	45.6 ± 1.0	20.1 ± 1.1	32.1 ± 0.5*
	300	37.5 ± 1.2	19.3 ± 1.1	40.3 ± 1.4*
Hydroxytyrosol+ α-Lipoic Acid	100	43.6 ± 1.1	21.2 ± 2.0	33.6 ± 1.2*
	150	42.7 ± 1.3	20.1 ± 1.0	35.7 ± 1.1*
	300	35.4 ± 0.3	21.0 ± 1.1	42.1 ± 1.0*
Ester **8**	100	31.3 ± 1.0	17.7 ± 1.0	49.7 ± 1.2*,**
	150	29.3 ± 1.1	18.1 ± 1.3	50.5 ± 1.1*,**
	300	28.2 ± 0.9	14.0 ± 1.2	56.8 ± 1.1*,**

Results were expressed as the mean ± S.D. of triplicates. *,**$P < 0.05$; *P values were calculated comparing the experimental results to control results; **P values were calculated comparing ester **8** results to results obtained with hydroxytyrosol and α-lipoic acid, either singularly or in combination.

4.1.1. Synthesis depicted in Scheme 1

4.1.1.1. Step (a). Tyrosol (0.5 mmol, 69 mg) was solubilised in dimethyl carbonate (3 ml) and DBU (0.6 mmol) was added. The mixture was kept under stirring at 90 °C for 7.5 h. At the end of the reaction, the solvent was evaporated under vacuum. The residue was solubilised with ethyl acetate (10 ml) and treated with a solution of 1 N HCl (5 ml). The aqueous phase was extracted with ethyl acetate (3 × 10 ml). The organic phases were washed with a saturated solution of NaCl (10 ml) and dried over Na_2SO_4. Following evaporation of the solvent, 4-hydroxyphenethyl methyl carbonate **3** was isolated as oil in quantitative yield. Spectroscopic data were accordant with those previously reported [33].

4.1.1.2. Step (b). 4-Hydroxyphenethyl methyl carbonate **3** (0.5 mmol, 76 mg) was dissolved in methanol (15 ml), then IBX (0.6 eq.) was added at 0 °C for 2.5 h. At the end, water (15 ml) and $Na_2S_2O_4$ (1.2 eq.) were added and the solution was stirred for 5–10 min. Following evaporation of the solvent under vacuum, the residue was solubilised with ethyl acetate (10 ml) and treated with a saturated solution of $NaHCO_3$ (10 ml). The aqueous phase was extracted with ethyl acetate (3 × 10 ml). The organic phases were washed with a saturated solution of NaCl (10 ml) and dried over Na_2SO_4. Following evaporation of the solvent and chromatographic purification on silica gel (eluent: ethyl acetate/hexane = 1:3), 3,4-dihydroxyphenethyl methyl carbonate **4** was isolated in 80% yield (85 mg). Spectroscopic data were accordant with those previously reported [33].

4.1.1.3. Step (c). Under argon atmosphere and in the dark, 2,2-dimethoxypropane (4.5 mmol) and camphorsulphonic acid (0.1 mmol) were added to a solution of 3,4-dihydroxyphenethyl methyl carbonate **4** (0.5 mmol, 106 mg) in anhydrous $CHCl_3$ (6 ml). The solution was kept at reflux for 48 h. At the end, the mixture was neutralised with a saturated solution of $NaHCO_3$ and the resulting aqueous phases were extracted with chloroform (3 × 10 ml). The combined organic extracts were dried over Na_2SO_4 and evaporated in vacuum. The crude residue was purified on silica gel (eluent: ethyl acetate/hexane = 1:3) to give 2-(2,2-dimethyl-1,3-benzo[1,3]dioxol-5-yl)ethyl methyl carbonate **5** in 90% yield (113 mg). Spectroscopic data were accordant with those previously reported [33].

4.1.1.4. Step (d). 2-(2,2-Dimethylbenzo[1,3]dioxol-5-yl)ethyl methyl carbonate **5** (0.5 mmol, 126 mg) was solubilised in DMF/water = 4:1 (5 ml), then potassium carbonate (1.0 mmol) was added. The mixture was stirred at 80 °C for 24 h. The reaction was monitored by thin layer chromatography. At the end, DMF was evaporated under vacuum. The residue was solubilised with ethyl acetate (10 ml) and treated with a solution of 1 N HCl (5 ml). The aqueous phase was extracted with ethyl acetate (3 × 10 ml). The organic phases were washed with a saturated solution of NaCl (10 ml) and dried over Na_2SO_4. Following evaporation of the solvent and chromatographic purification on silica gel (eluent: ethyl acetate/hexane = 1:5), 2-(2,2-dimethylbenzo[1,3]dioxol-5-yl)ethanol **6** was isolated in quantitative yield (97 mg). Spectroscopic data were accordant with those previously reported [34].

4.1.1.5. Step (e). Under nitrogen flow, 2-(2,2-dimethylbenzo[1,3]dioxol-5-yl)ethanol **6** (0.1 mmol, 38 mg) was dissolved in dichloromethane (5 ml). α-Lipoic acid **2** (0.12 mmol) and DMAP (0.3 mmol) were then added at room temperature. After cooling at 0 °C, a solution of DCC (0.12 mmol) in dichloromethane (2 ml) was added dropwise for 1 h. L-Cysteine (0.5%) was added to the solution as polymerization inhibitor. The reaction was kept at room temperature for 24 h. At the end, the mixture was filtered. Following evaporation of the solvent under vacuum, the residue was solubilised with ethyl acetate (5 ml) and treated with a solution of 1 N HCl (1 ml). The aqueous phase was extracted with ethyl acetate (3 × 5 ml). The organic phases were washed with a saturated solution of NaCl (5 ml) and dried over Na_2SO_4. Following chromatographic purification of the reaction mixture on silica gel (eluent: ethyl acetate/hexane = 1:7), 2-(2,2-dimethylbenzo[1,3]dioxol-5-yl)ethyl 5-(1,2-dithiolan-3-yl)pentanoate **7** was isolated in 90% yield (34 mg). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.32–1.64 (m, 6H, 3 × CH_2), 1.55 (s, 6H, 2 × CH_3), 1.75–1.84 (m, 1H, CH_2), 2.21 (t, 2H, J = 7.4 Hz, CH_2), 2.31–2.37 (m, 1H, CH_2), 2.72 (t, 2H, J = 7.0 Hz, CH_2), 2.99–3.10 (m, 2H, CH_2), 3.37–3.49 (m, 1H, CH), 4.12 (t, 2H, J = 7.0 Hz, CH_2), 6.50–6.57 (m, 3H, Ph). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 25.6, 25.7, 28.6, 33.7, 34.0, 34.4, 38.4, 40.1, 56.3, 65.8, 108.0, 109.0, 117.7, 121.1, 130.7, 146.0, 147.4, 173.7. HR-MS m/z: 382.5393 (M^+). Anal. Calcd. for $C_{19}H_{26}O_4S_2$: C, 59.66; H, 6.85; O, 16.73; S, 16.76. Found: C, 59.56; H, 6.80; O, 16.78; S, 16.86.

4.1.1.6. Step (f). 2-(2,2-Dimethylbenzo[1,3]dioxol-5-yl)ethyl 5-(1,2-dithiolan-3-yl)pentanoate **7** (0.1 mmol, 38 mg) was solubilised in a mixture of acetonitrile/water = 4:1 (5 ml), then p-toluensulphonic acid (0.1 mmol) was added. The mixture was stirred at 80 °C for 24 h. The reaction was monitored by thin layer chromatography. At the end of the reaction, DMF was evaporated under vacuum. The residue was solubilised with ethyl acetate (5 ml) and treated with a saturated solution of $NaHCO_3$ (5 ml). The aqueous phase was extracted with ethyl acetate (3 × 5 ml). The organic phases were

washed with a saturated solution of NaCl (5 ml) and dried over Na_2SO_4. Following evaporation of the solvent, 3,4-dihydroxyphenethyl 5-(1,2-dithiolan-3-yl)pentanoate **8** was isolated in 62% yield (21 mg, oil). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.23–1.37 (m, 2H, CH_2), 1.48–1.62 (m, 4H, 2 × CH_2), 1.78–1.91 (m, 1H, CH_2), 2.29 (t, 2H, J = 7.0 Hz, CH_2), 2.33–2.48 (m, 2H, CH_2), 2.80 (t, 2H, J = 7.0 Hz, CH_2), 3.00–3.15 (m, 2H, CH_2), 3.43–3.51 (m, 1H, CH), 4.23 (t, 2H, J = 7.0 Hz, CH_2), 6.51 (dd, 1H, J_1 = 8.0 Hz, J_2 = 2.1, Ph), 6.64 (d, 1H, J = 2.1 Hz, Ph), 6.69 (d, 1H, J = 8.0 Hz, Ph). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 24.6, 28.6, 29.6, 34.4, 34.5, 38.4, 40.2, 56.3, 65.1, 115.0, 115.6, 120.6, 129.8, 142.9, 144.2, 173.8. HR-MS m/z: 342.4755 (M^+). Anal. Calcd. for $C_{16}H_{22}O_4S_2$: C, 56.11; H, 6.47; O, 18.69; S, 18.73. Found: C, 56.20; H, 6.55; O, 18.65; S, 18.60.

4.1.2. Synthesis depicted in Scheme 2

4.1.2.1. Step (a). See the procedure described in Section 4.1.1.1. Reaction time was 24 h. Following evaporation of the solvent, 4-methoxyphenethyl methyl carbonate **9** was isolated as an oil in quantitative yield. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 2.89 (t, 2H, J = 7.1 Hz, CH_2), 3.73 (s, 3H, $COOCH_3$), 3.75 (s, 3H, OCH_3), 4.27 (t, 2H, J = 7.1 Hz, CH_2), 6.82 (d, 2H, J = 8.6 Hz, Ph), 7.11 (d, 2H, J = 8.6 Hz, Ph). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 34.3, 54.7, 55.2, 68.6, 114.0, 114.1, 129.2, 129.3, 129.9, 156.0, 158.4. HR-MS m/z: 210.2265 (M^+). Anal. Calcd. for $C_{11}H_{14}O_4$: C, 62.85; H, 6.71; O, 30.44. Found: C, 62.92; H, 6.68; O, 30.40.

4.1.2.2. Step (b). 4-Methoxyphenethyl methyl carbonate **9** (0.5 mmol, 105 mg) was solubilised in THF (10 ml), then a 1 N solution of NaOH (2 ml) was added. The mixture was stirred at room temperature and monitored by thin layer chromatography. After 24 h, the solvent was evaporated under vacuum. The residue was neutralised with a 1 N solution of HCl and the aqueous phase was extracted with ethyl acetate (3 × 10 ml). The organic phases were washed with a saturated solution of NaCl (10 ml) and dried over Na_2SO_4. Following evaporation of the solvent, 2-(4-methoxyphenyl)ethanol **10** was isolated in quantitative yield (0.5 mmol, 76 mg). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 2.79 (t, 2H, J = 6.5 Hz, CH_2), 3.77 (s, 3H, OCH_3), 3.80 (t, 2H, J = 6.6 Hz, CH_2), 6.84 (d, 2H, J = 8.7 Hz, Ph), 7.13 (d, 2H, J = 8.6 Hz, Ph). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 38.3, 55.3, 63.8, 114.0, 129.8, 129.9, 130.4, 158.3. HR-MS m/z: 152.1904 (M^+). Anal. Calcd. for $C_9H_{12}O_2$: C, 71.03; H, 7.95; O, 21.03. Found: C, 70.89; H, 8.01; O, 21.10.

4.1.2.3. Step (c). See the procedure described in Section 4.1.1.5. Following chromatographic purification of the reaction mixture on silica gel (eluent: ethyl acetate/hexane = 1:7), 4-methoxyphenethyl 5-(1,2-dithiolan-3-yl)pentanoate **11** was isolated in 92% yield. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.24–1.36 (m, 2H, CH_2), 1.43–1.73 (m, 4H, 2 × CH_2), 1.76–1.82 (m, 1H, CH_2), 2.23 (t, 2H, J = 7.0 Hz, CH_2), 2.32–2.38 (m, 1H, CH_2), 2.76 (t, 2H, J = 7.0 Hz, CH_2), 3.01–3.07 (m, 2H, CH_2), 3.42–3.49 (m, 1H, CH), 3.67 (s, 2H, OCH_3), 4.13 (t, 2H, J = 7.0 Hz, CH_2), 6.73 (d, 2H, J = 8.6 Hz, Ph), 7.02 (d, 2H, J = 8.6, Ph). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 24.6, 28.6, 33.7, 34.0, 34.2, 38.4, 40.2, 55.2, 56.3, 65.0, 113.8, 113.9, 129.8, 129.9, 158.3, 173.5. HR-MS m/z: 340.5027 (M^+). Anal. Calcd. for $C_{17}H_{24}O_3S_2$: C, 59.97; H, 7.10; O, 14.10; S, 18.83. Found: C, 60.05; H, 7.12; O, 14.05; S, 18.78.

4.1.2.4. Step (d). 4-Methoxyphenethyl 5-(1,2-dithiolan-3-yl)pentanoate **11** (0.1 mmol, 34 mg) was dissolved in dry THF (2 ml), then the mixture was cooled at −15 °C and a solution of 1 M boron tribromide in dichloromethane (0.2 mmol) was added. After 2 h, the solvent was evaporated under vacuum, and the residue was solubilised with ethyl acetate (5 ml) and treated with a saturated solution of $NaHCO_3$ (2 ml). The aqueous phase was extracted with ethyl acetate (3 × 5 ml). The organic phases were washed with a saturated solution of NaCl (5 ml) and dried over Na_2SO_4. Following evaporation of the solvent and chromatographic purification on silica gel (eluent: ethyl acetate/hexane = 1:2), 4-hydroxyphenethyl 5-(1,2-dithiolan-3-yl)pentanoate **12** was isolated in 84% yield (27 mg, oil). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.30–1.43 (m, 2H, CH_2), 1.50–1.67 (m, 4H, 2 × CH_2), 1.79–1.92 (m, 1H, CH_2), 2.25 (t, 2H, J = 7.0 Hz, CH_2), 2.32–2.48 (m, 1H, CH_2), 2.80 (t, 2H, J = 7.0 Hz, CH_2), 3.05–3.14 (m, 2H, CH_2), 3.42–3.56 (m, 1H, CH), 4.19 (t, 2H, J = 7.0 Hz, CH_2), 6.71 (d, 2H, J = 8.5 Hz, Ph), 7.00 (d, 2H, J = 8.5 Hz, Ph). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 24.6, 28.6, 29.6, 34.0, 34.2, 38.4, 40.2, 56.3, 65.2, 115.2, 115.3, 128.8, 129.8, 129.9, 155.2, 173.7. HR-MS m/z: 326.4761 (M^+). Anal. Calcd. for $C_{16}H_{22}O_3S_2$: C, 58.86; H, 6.79; O, 14.70; S, 19.64. Found: C, 58.75; H, 6.88; O, 14.65; S, 19.72.

4.1.2.5. Step (e). Aromatic hydroxylation of 4-hydroxyphenethyl 5-(1,2-dithiolan-3-yl)pentanoate **12** was performed according to the procedure described in Section 4.1.1.2. Following chromatographic purification of the reaction mixture on silica gel (eluent: ethyl acetate/hexane = 1:7), 3,4-dihydroxyphenethyl 5-(1,2-dithiolan-3-yl)pentanoate **8** was recovered in 80% yield (27 mg).

4.2. Antiproliferative activity

4.2.1. Test compounds

Hydroxytyrosol, α-lipoic acid, and ester **8** were dissolved in 0.1% dimethyl sulphoxide (DMSO, Sigma) at final concentrations of 100, 150, and 300 μM. For controls, the equivalent volume of 0.1% DMSO alone was added.

4.2.2. Test system and culture conditions

The human colorectal adenocarcinoma HT-29 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in Dulbecco's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 0.3 mg/ml L-glutamine, in a humidified incubator at 37 °C, 5% CO_2.

4.2.3. Viability assessment

Cell viability was assessed by the trypan blue dye exclusion assay.

4.2.4. Proliferation assay

Cells (2 × 10^3 cells/ml) were cultured in triplicates in 96-well plates with the different compounds at the indicated concentrations at 37 °C for 24 and 48 h. Proliferation was assessed using a BrdU-ELISA kit (Roche Diagnostics) according to the manufacturer's instructions. Proliferative results are reported as the net absorbance values: absorbance at λ = 450 nm of pulse-labelled cells − absorbance at λ = 450 nm of unlabelled cells.

4.2.5. Cell cycle analysis

Cell cycle was analysed by flow cytometry. Briefly, cells (2 × 10^5) were seeded in 6-well culture plates and maintained in a humidified atmosphere at 37 °C, 5% CO_2 for 24 h. Cell cultures were then incubated with native or synthetic compounds at the indicated concentrations. After 24 h, the cells were harvested, washed, resuspended in a hypotonic buffer, and incubated with 50 μg/ml propidium iodide at 4 °C for 1 h in the dark. The cells were subsequently analysed using a FACScalibur (Becton Dickinson, San Jose, CA, USA); at least 5 × 10^4 events were acquired and analysed using CELLQuest software.

4.2.6. Statistics

Results from the proliferation and cell cycle experiments were evaluated by the one-way ANOVA test; differences between control

and treated cells were determined by Tukey's comparison test. For proliferation and cell cycle analysis, five independent experiments were performed. Data were expressed as the mean ± S.E.

Acknowledgements

The authors would like to thank the Ministero dell'Università e della Ricerca Scientifica e Tecnologica and the University of Tuscia, Viterbo (Italy) for financial support. The authors are grateful to Dr. M. Barontini for the preparation of some samples.

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