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Selective Cytotoxic Activity of New Lipophilic Hydroxytyrosol Alkyl Ether Derivatives

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ABSTRACT: Recent data suggest that hydroxytyrosol, a phenolic compound of virgin olive oils, has anticancer activity. This communication reports the synthesis of decyl and hexadecyl hydroxytyrosyl ethers, as well as the cytotoxic activity of hydroxytyrosol and a series of seven hydroxytyrosol alkyl ether derivatives against A549 lung cancer cells and MRC5 non-malignant lung fibroblasts. Hydroxytyrosyl dodecyl ether (HTDE) showed the highest selective cytotoxicity, and possible mechanisms of action were investigated; results suggest that HTDE can moderately inhibit glycolysis, induce oxidative stress, and cause DNA damage in A549 cells. The combination of HTDE with the anticancer drug 5-fluorouracil induced a synergistic cytotoxicity in A549 cancer cells but not in non-malignant MRC5 cells. HTDE also displayed selective cytotoxicity against MCF7 breast cancer cells versus MCF10 normal breast epithelial cells in the 1−30 μM range. These results suggest that the cytotoxicity of HTDE is more potent and selective than that of parent compound hydroxytyrosol.

KEYWORDS: *olive oil, lipophilic derivatives, phenolic compounds, cancer, anticancer, cytotoxic*

INTRODUCTION

Epidemiological studies suggest that a Mediterranean diet may reduce the risk of developing degenerative pathologies such as cardiovascular diseases and cancer.[1−4] Since olive oil is the main source of fat in the Mediterranean diet, the number of reports describing the beneficial properties of olive oil has notably increased in recent years. This olive oil popularity has mainly been attributed to its high content of monounsaturated fatty acids and to its richness in phenolic compounds.[3−9]

The presence of phenolic compounds in olive oil has attracted much attention due to their known biological activities and health effects. Particular attention has been placed on 3,4-dihydroxyphenylethanol (hydroxytyrosol), an *o*-diphenolic compound that is present in virgin olive oil as secoiridoid derivatives or acetate esters and has shown a variety of pharmacological activities.[10−15] Accumulating preclinical evidence suggests that hydroxytyrosol has cancer chemopreventive and chemotherapeutic potential. In vitro experiments have shown that hydroxytyrosol inhibits proliferation and induces apoptosis in a variety of cancer cell lines from different origin, including breast, colon, and leukemia cell lines.[16−20] Several mechanisms of action have been shown to participate in its cytotoxic activity, such as cell cycle arrest; cytochrome *c* release; activation of caspase 3, c-jun, or bcl-2; and inhibition of CDK6, HER2, fatty acid synthase, or PI3K/Akt/NF-kappa B pathway (reviewed in ref 11). Recent animal experiments have revealed that hydroxytyrosol can also inhibit cancer cell growth in vivo.[21]

Our previous work reported the syntheses of lipophilic hydroxytyrosol derivatives in the form of esters[22] and ethers[23] and the evaluation of their antioxidant activity in relation to that of hydroxytyrosol.[24−26] These studies revealed that the introduction of an acyl or alkyl side chain in the hydroxytyrosol structure did not reduce, or even improved, the antioxidant capacity of hydroxytyrosol. We have studied the absorption, metabolism, and digestive stability of these compounds[27−29] and have also observed that some of these hydroxytyrosol derivatives exhibit antiplatelet, anti-inflammatory, and neuroprotective effects.[30,31] In this article we report the synthesis of two new alkyl derivatives of hydroxtyrosol, as well as the evaluation of the cytotoxic activity on cancer and non-malignant cells of hydroxytyrosol and a series of its ether derivatives (ethyl, butyl, hexyl, octyl, decyl, dodecyl, and hexadecyl ethers). Interestingly, hydroxytyrosol isolated from olive oil waste waters (OOWW) has been used for the preparation of these compounds to give an added value to this type of residue.

MATERIALS AND METHODS

Chemicals. Hydroxytyrosol was obtained from olive oil waste waters as described elsewhere.[23] Hydroxytyrosol alkyl ether derivatives were prepared from hydroxytyrosol as described below. Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (MnTMPyP) was purchased from Biomol International. All other compounds used in this work were obtained from Sigma. All compounds were dissolved in DMSO and added to the cells after appropriate dilutions in cell culture medium. Final DMSO concentrations were never higher than 0.3%.

Cell Lines. The human A549 lung cancer cell line, the human embryo lung fibroblastic MRC5 cell line, and the human MCF7 breast adenocarcinoma cell line were maintained in DMEM supplemented with 2 mM glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum. The human MCF10 breast epithelial cell line (kindly provided by Dr. D. Ruano and Dr. P. Daza) was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 10 μg/mL insulin, and 500 ng/mL hydrocortisone (95%)/horse serum (5%). To study the

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possible DNA damage response induced by the tested compounds, the following parental and DNA repair-deficient cell lines were used: AA8, parental Chinese hamster ovary cells; V3-3, AA8 cells mutated in XRCC7 (DNA-PK), nonhomologous end joining (NHEJ) deficient; KO40, AA8 cells mutated in FANCG, Fanconi anemia (FA) deficient; VC8, V79 Chinese hamster lung cells mutated in BRCA2, homologous recombination (HR) deficient; VC8B2, VC8 cells complemented with human BRCA2 (HR proficient); EM9-V, AA8 cells mutated in XRCC1 (DNA ligase III), base excision repair (BER) deficient; EM9-XH, EM9 cells complemented with XRCC1 (BER proficient); HCT-116, human colon cancer cells mutated in MLH1, mismatch repair (MMR) deficient; and HCT-116 + c3, HCT-116 cells complemented with chromosome 3 (with MLH1 gene; MMR proficient).[32,33] These DNA repair-deficient cell lines, kindly provided by Dr. Thomas Helleday, were cultured in DMEM. All cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Cell culture reagents were obtained from Life Technologies.

Preparation of Ether Derivatives of Hydroxytyrosol. Hydroxytyrosyl alkyl ethers (**10**−**16**) were prepared from hydroxytyrosol (**1**) using a method previously described by us.[23] The following compounds have been synthesized for the first time:

1,2-Bis(benzyloxy)-4-(2′-decyloxyethyl)benzene (7). White solid (86% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.37 (m, 10H, 2 × Ph), 6.96 (d, J = 2.0 Hz, 1H, H_4), 6.92 (d, J = 8.2 Hz, 1H, H_7), 6.71 (dd, 1H, H_8), 5.07 (s, 2H, CH_2Ph in pos. 5), 5.06 (s, 2H, CH_2Ph in pos. 6), 3.48 (t, J = 6.9 Hz, 2H, H_1), 3.32 (t, J = 6.5 Hz, 2H, $H_{1'}$), 2.68 (t, 2H, H_2), 1.43 (m, 2H, $H_{2'}$), 1.23 (m, 14H, $H_{3'}$−$H_{9'}$), 0.83 (t, 3H, $H_{10'}$). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm 148.2 (C_5), 146.6 (C_6), 137.4 and 137.3 (C_{ipso}, Bn groups), 132.2 (C_3), 128.2−127.4 ($C_{3''}$, $C_{4''}$, and $C_{5''}$, Bn groups), 121.2 (C_8), 115.4 (C_4), 114.6 (C_7), 70.9 (C_1), 70.2 (CH$_2$Ph in pos. 6), 70.1 (CH$_2$Ph in pos. 5), 69.8 ($C_{1'}$), 35.0 (C_2), 29.1 ($C_{2'}$), 29.0−28.6 ($C_{4'}$−$C_{7'}$), 25.6 ($C_{3'}$), 31.2 ($C_{8'}$), 22.0 ($C_{9'}$), 13.8 ($C_{10'}$). Anal. Calcd for $C_{32}H_{42}O_3$: C, 80.97; H, 8.92. Found: C, 80.85; H, 8.78.

1,2-Bis(benzyloxy)-4-(2′-hexadecyloxyethyl)benzene (9). White solid (86% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.37 (m, 10H, 2 × Ph), 6.96 (d, J = 2.0 Hz, 1H, H_4), 6.93 (d, J = 8.2 Hz, 1H, H_7), 6.72 (dd, 1H, H_8), 5.08 (s, 2H, CH_2Ph in pos. 5), 5.07 (s, 2H, CH_2Ph in pos. 6), 3.49 (t, J = 7.0 Hz, 2H, H_1), 3.33 (t, J = 6.5 Hz, 2H, $H_{1'}$), 2.69 (t, 2H, H_2), 1.45 (m, 2H, $H_{2'}$), 1.23 (m, 26H, $H_{3'}$−$H_{15'}$), 0.84 (t, 3H, $H_{16'}$). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm 148.1 (C_5), 146.6 (C_6), 137.4 and 137.3 (C_{ipso}, Bn groups), 132.2 (C_3), 128.2−127.4 ($C_{3''}$, $C_{4''}$ y $C_{5''}$, Bn groups), 121.3 (C_8), 115.5 (C_4), 114.7 (C_7), 70.9 (C_1), 70.2 (CH$_2$Ph in pos. 6), 70.1 (CH$_2$Ph in pos. 5), 69.8 ($C_{1'}$), 35.0 (C_2), 31.2 ($C_{14'}$), 29.1 ($C_{2'}$), 29.0−28.6 ($C_{4'}$−$C_{13'}$), 25.6 ($C_{3'}$), 22.0 ($C_{15'}$), 13.8 ($C_{16'}$). Anal. Calcd for $C_{38}H_{54}O_3$: C, 81.67; H, 9.74. Found: C, 81.27; H, 9.35.

4-(2′-Decyloxyethyl)benzene-1,2-diol (14). Colorless oil (83% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 8.63 (s, 1H, *OH* in pos. 6), 8.56 (s, 1H, *OH* in pos. 5), 6.59 (d, J = 8.0 Hz, 1H, H_7), 6.57 (d, J = 2.1 Hz, 1H, H_4), 6.43 (dd, 1H, H_8), 3.44 (t, J = 7.2 Hz, 2H, H_1), 3.33 (t, J = 6.6 Hz, 2H, $H_{1'}$), 2.58 (t, 2H, H_2), 1.41 (m, 2H, $H_{2'}$), 1.24 (m, 14H, $H_{3'}$−$H_{9'}$), 0.84 (t, 3H, $H_{10'}$). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm 144.8 (C_5), 143.3 (C_6), 129.7 (C_3), 119.3 (C_8), 116.1 (C_4), 115.3 (C_7), 71.4 (C_1), 69.9 ($C_{1'}$), 34.9 (C_2), 29.1 ($C_{2'}$), 29.0−28.6 ($C_{4'}$−$C_{7'}$), 25.6 ($C_{3'}$), 31.2 ($C_{8'}$), 22.0 ($C_{9'}$), 13.8 ($C_{10'}$). Anal. Calcd for $C_{18}H_{30}O_3$: C, 73.43; H, 10.27. Found: C, 73.41; H, 10.39.

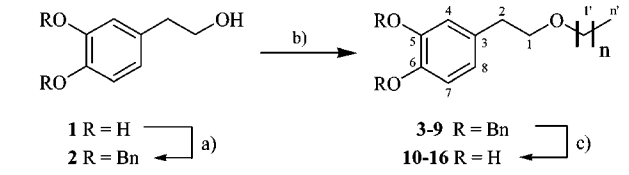
4-(2′-Hexadecyloxyethyl)benzene-1,2-diol (16). White solid (83% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 8.59 (s, 2H, *OH* in pos. Five and 6), 6.60 (d, J = 8.0 Hz, 1H, H_7), 6.58 (d, J = 2.1 Hz, 1H, H_4), 6.43 (dd, 1H, H_8), 3.44 (t, J = 7.2 Hz, 2H, H_1), 3.33 (t, J = 6.6 Hz, 2H, $H_{1'}$), 2.59 (t, 2H, H_2), 1.45 (m, 2H, $H_{2'}$), 1.23 (m, 26H, $H_{3'}$−$H_{15'}$), 0.85 (t, 3H, $H_{16'}$). ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm 144.8 (C_5), 143.3 (C_6), 129.7 (C_3), 119.3 (C_8), 116.1 (C_4), 115.3 (C_7), 71.4 (C_1), 69.9 ($C_{1'}$), 35.0 (C_2), 31.2 ($C_{14'}$), 29.1 ($C_{2'}$), 29.0−28.6 ($C_{4'}$−$C_{13'}$), 25.6 ($C_{3'}$), 22.0 ($C_{15'}$), 13.8 ($C_{16'}$). Anal. Calcd for $C_{24}H_{42}O_3$: C, 76.14; H, 11.18. Found: C, 76.41; H, 10.71.

Cell Proliferation Assay. The MTT assay is a colorimetric technique for the quantitative determination of cell viability. It is based on the capability of viable cells to transform the MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a purple formazan dye. Exponentially growing cells were seeded into 96-well plates, and drugs were added 24 h later. Cells were exposed to the drugs for 48 h (except in the experiments with the parental and DNA repair-deficient cell lines, in which cells were exposured for 24 h and were then allowed to grow for additional 24 h in drug-free medium to let them repair the possible DNA damage induced by the drugs). After these 48 h, medium was removed, and 125 μL MTT (1 mg/mL in medium) was added to each well for 5 h. Then, 80 μL 20% SDS in 20 mM HCl was added to dissolve the insoluble purple formazan product, plates were incubated for 10 h at 37 °C, and optical densities were measured at 540 nm on a multi-well plate spectrophotometer reader. Cell viability was expressed as percentage in relation to controls. All data were averaged from at least three independent experiments and were expressed as mean ± standard error of the mean (SEM).

Inhibition of Glycolysis. Glycolysis inhibition was assessed by measuring concentrations of glucose (initial product of glycolysis) and lactate (final product of glycolysis) in control and treated cells. Briefly, 10^6 cells were exposed to the tested compounds for 8 h, and glucose and lactate concentrations were determined in cell supernatants by using the Accutrend Plus analyzer together with Accutrend glucose strips and BM-Lactate strips (Roche Diagnostics). After calibrating the instrument with glucose and lactate calibration strips, test strips were used to determine glucose and lactate levels via colorimetric-oxidase mediator reactions according to the manufacturer's instructions.[34] Results are expressed as percentage of lactate production and percentage of glucose consumption in relation to untreated cells and are shown as the mean ± standard error of the mean (SEM) of three independent experiments.

Comet Assay. The single-cell gel electrophoresis assay (comet assay) is a well established technique for DNA damage detection. The images obtained with this assay resemble a "comet" with a distinct head and tail; the head is composed of intact DNA, while the tail consists of damaged (single-strand or double-strand breaks) DNA. This assay has been described in detail by Singh et al.[35] We followed this protocol with minor modifications described previously.[36] Briefly, standard slides were immersed in 1% normal melting agarose at 55 °C, left to allow the agarose to solidify, and kept at 4 °C until use. After cell treatments, approximately 10,000 cells were mixed with 85 μL of low-melting agarose (LMA) at 37 °C. This mixture was rapidly pipetted onto the slides with the first agarose layer, spread using a coverslip, and kept at 4 °C for 8 min to allow the LMA to solidify. The coverslips were then removed, and a third layer of 100 μL of LMA at 37 °C was added, covered with a coverslip, and allowed to solidify at 4 °C for 8 min. After removal of the coverslips, cells were incubated in the dark for 1 h at 4 °C in a lysis solution containing 10 mM Tris-HCl, 2.5 M NaCl, 100 mM Na_2-EDTA, 0.25 M NaOH, 1% (v/v), Triton X-100, and 10% (v/v) DMSO, pH 12.0. To unwind the DNA, the slides were incubated for 20 min in an electrophoretic buffer containing 1 mM Na_2-EDTA and 300 mM NaOH, pH 12.8. Electrophoresis was carried out at 1 V/cm for 20 min. After neutralization with 3 × 5 min washes of 0.4 M Tris-HCl, pH 7.5, to remove alkali and detergent, cells were stained with the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (mounting medium for fluorescence H-1000, Vector Laboratories, Peterborough, U.K.). Images of 50 randomly selected cells from each sample were analyzed using the "comet score" software. DNA damage was calculated for each comet and was expressed as percent of DNA in the tail and as tail moment (defined as the product of the tail length and the fraction of total DNA in the tail). Data were averaged from three independent experiments and were expressed as mean ± standard error of the mean (SEM).

Statistical Analysis. For statistical analysis we used the t test (paired, two-tailed). A P-value >0.05 is not considered statistically significant and is not represented by any symbol. A P-value <0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), a P-value <0.01 is indicated with a double asterisk (**), and a P-value <0.001 is indicated with a triple asterisk (***). When the cytotoxicity of a compound was evaluated against two cell lines, the statistical analysis was carried out to compare the cytotoxicity of a particular concentration of the compound between both cell lines.



Side chain	n	R=Bn	R=H
Ethyl	1	**3**	**10**
Butyl	3	**4**	**11**
Hexyl	5	**5**	**12**
Octyl	7	**6**	**13**
Decyl	9	**7**	**14**
Dodecyl	11	**8**	**15**
Hexadecyl	15	**9**	**16**

Figure 1. Synthetic procedure of alkyl hydroxytyrosyl ether derivatives **10**−**16**.

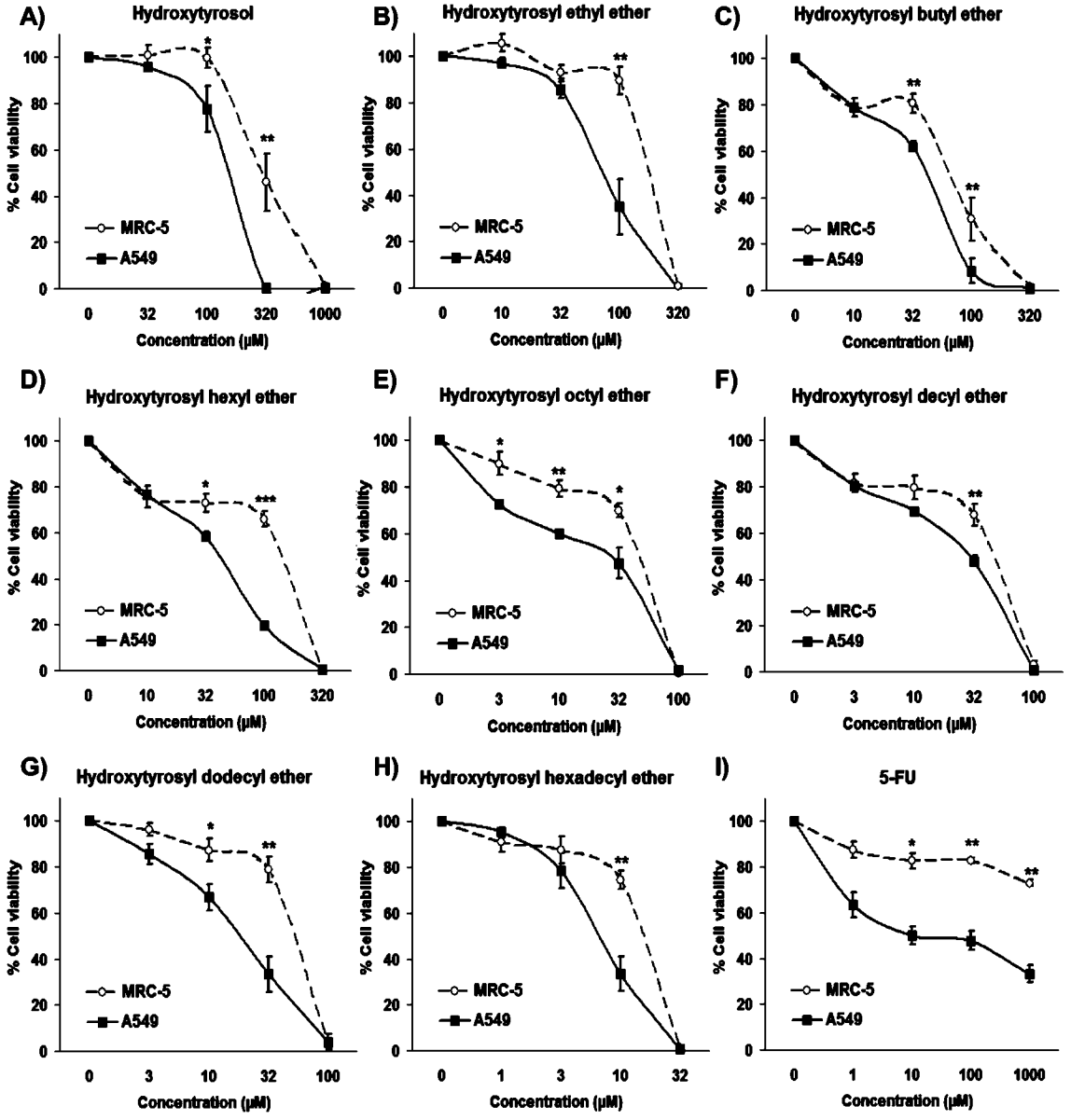


Figure 2. Selective cytotoxicity of hydroxytyrosol and several alkyl hydroxytyrosyl ether derivatives (**10**−**16**). The percentage of cell viability (mean ± SEM) in A549 cancer cells and MRC5 normal cells exposed for 48 h to hydroxytyrosol (**1**), its alkyl ether derivatives (**10**−**16**), and 5-fluorouracil (5-FU) was determined with the MTT assay.

RESULTS AND DISCUSSION

Ethyl (C2, **10**), butyl (C4, **11**), hexyl (C6, **12**), octyl (C8, **13**), decyl (C10, **14**), dodecyl (C12, **15**), and hexadecyl (C16, **16**) hydroxytyrosyl ethers were prepared from hydroxytyrosol (**1**) as shown in Figure 1, following a method previously described.[23] Decyl (**14**) and hexadecyl (**16**) hydroxytyrosol ethers were synthesized for the first time and were obtained in good yields. Spectral data of these compounds are in good agreement with the proposed structures.

The MTT assay was used to assess the cytotoxic activity of hydroxytyrosol (**1**) and its alkyl ethers (**10**−**16**) against A549 lung cancer cells and MRC5 non-malignant lung fibroblasts. The results, represented in Figure 2, show a direct relationship between the lipophilic nature of these compounds and their

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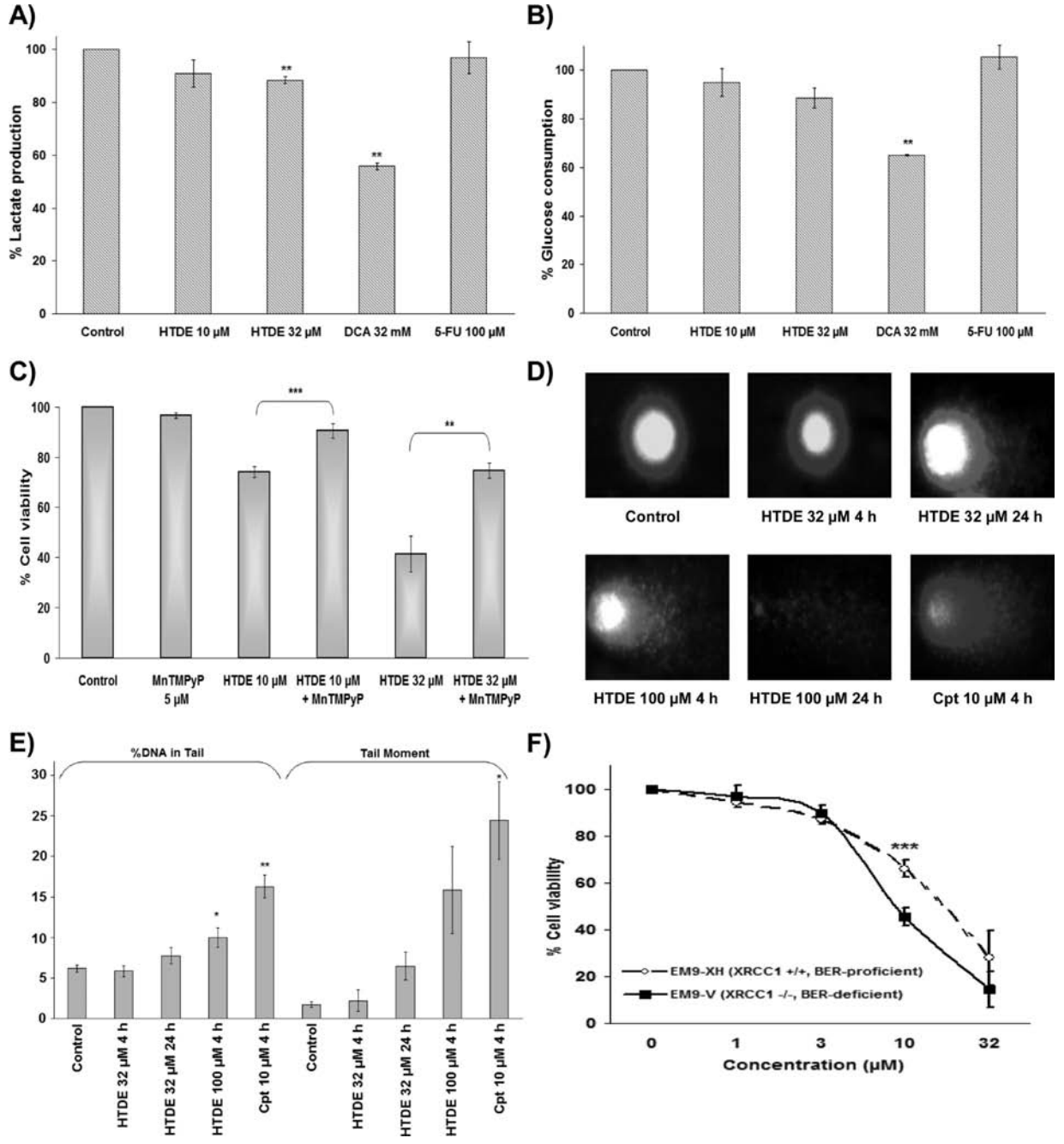

Figure 3. Possible mechanisms of action involved in the cytotoxicity of hydroxytyrosyl dodecyl ether (HTDE, **15**). (A) Percentage of lactate produced by cells exposed for 8 h to HTDE (**15**), dichloroacetate (DCA), and 5-fluorouracil (5-FU) in relation to untreated cells (control). (B) Percentage of glucose consumed by cells exposed for 8 h to HTDE, DCA, and 5-FU in relation to untreated cells. (C) Prevention of HTDE-induced cell death in A549 cells by the superoxide dismutase mimetic MnTMPyP (MTT assay); A549 cells were exposed to HTDE for 48 h in the presence and absence of MnTMPyP (added 1 h before HTDE). (D) Representative photographs of untreated cells, of cells treated with the DNA damaging agent camptothecin (cpt), and of cells exposed for 4 and 24 h to HTDE at 32 μM and 100 μM (comet assay). (E) Quantification of DNA damage (comet assay) expressed as percent of DNA damage in tail and as tail moment (tail length × percentage of DNA in the tail). (F) Cells mutated in XRCC1 (deficient in base excision repair) are more sensible than parental cells to the cytotoxic activity of HTDE. Both cell lines were treated with several concentrations of HTDE for 24 h, and after a recovery period of 24 h, cell viability was estimated with the MTT assay.

cytotoxic activity on A549 lung cancer cells. All compounds had certain selectivity for the cancer cell line, i.e., specific concentrations of these compounds induced a higher cytotoxicity in A549 cancer cells than in MRC5 normal cells. This selective cytotoxicity, however, was lower than that of the anticancer drug 5-fluorouracil (5-FU; positive control). Several compounds, including curcumin (a natural product currently undergoing clinical development for treatment of various cancers), the lipophilic compound β-carotene, and sodium hypochlorite (the principal constituent of bleach), were also screened against these two cell lines and showed very little or no selective cytotoxicity (negative controls). IC_{50} values were calculated for all tested compounds and are presented in Table 1.

Although hydroxytyrosyl hexadecyl ether was the most cytotoxic alkyl ether derivative in A549 cells, hydroxytyrosyl dodecyl ether (HTDE) showed the highest selective cytotoxic

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Table 1. IC_{50} Values of Hydroxytyrosol, Several Alkyl Hydroxytyrosyl Ether Derivatives, 5-Fluorouracil (Positive Control), and Several Compounds (Negative Controls) in A549 Lung Cancer Cells and MRC5 Normal Lung Cells (MTT Assay)

	IC_{50} (mean ± SEM; μM)		
compound	MRC5	A549	*P*-value
hydroxytyrosol	379.7 ± 54.9	147.0 ± 16.5	0.0008
hydroxytyrosyl ethyl ether	166.5 ± 7.8	86.1 ± 14.4	0.0002
hydroxytyrosyl butyl ether	78.9 ± 13.3	43.8 ± 3.4	0.0098
hydroxytyrosyl hexyl ether	131.6 ± 6.0	41.2 ± 2.2	0.0001
hydroxytyrosyl octyl ether	44.0 ± 1.5	30.0 ± 4.5	0.0367
hydroxytyrosyl decyl ether	47.1 ± 6.1	28.2 ± 2.4	0.0097
hydroxytyrosyl dodecyl ether	49.0 ± 4.3	19.9 ± 4.6	0.0164
hydroxytyrosyl hexadecyl ether	14.7 ± 0.8	7.2 ± 1.3	0.0056
5-fluorouracil	>1000	4.8 ± 0.5	
curcumin	16.3 ± 0.3	16.5 ± 0.7	0.8255
β-carotene	311.9 ± 1.3	289.0 ± 2.3	0.0049
sodium hypochlorite	0.8 ± 0.2 mM	1.7 ± 0.1 mM	0.0373

activity (Figure 2). The IC_{50} value in the A549 cancer cell line was 2.46 times lower than in the normal MRC5 cell line for HTDE and 2.04 times for hydroxytyrosyl hexadecyl ether. Since HTDE was the most selective hydroxytyrosol alkyl ether derivative, our next goal was to evaluate possible mechanisms involved in this selective cytotoxicity. We initially tested the ability of HTDE to inhibit glycolysis. Evidence suggest that cancer cells have higher reliance on glycolysis for their survival than normal cells, and that the inhibition of glycolysis may cause selective anticancer effects.[37−39] The possible inhibition of glycolysis by HTDE was assessed by measuring concentrations of glucose (initial product of glycolysis) and lactate (final product of glycolysis) in control and treated A549 lung cancer cells. The results suggest that HTDE (10 and 32 μM) induced a modest reduction in lactate production and glucose consumption, although only the reduction in lactate production induced by HTDE at 32 μM was statistically significant (Figure 3A,B). This inhibition of glycolysis was lower than that induced by the glycolysis inhibitor dichloroacetate. 5-FU did not reduce either lactate production or glucose consumption (negative control). The percentages of lactate production and glucose consumption (mean ± SEM) induced by these compounds in relation to untreated cells were, respectively, HTDE 10 μM (90.97 ± 5.17; 95.10 ± 5.70), HTDE 32 μM (88.49 ± 1.18; 88.78 ± 4.15), dichloroacetate 32 mM (55.89 ± 1.33; 65.00 ± 0.39), and 5-FU 100 μM (96.95 ± 6.11; 99.99 ± 5.00).

We next evaluated whether the formation of reactive oxygen species (ROS) played a role in the cytotoxicity of HTDE. Accumulating data suggest that cancer cells have higher basal levels of ROS than non-malignant cells and that the induction of a specific increase in ROS levels by pro-oxidant agents may lead to cytotoxic concentrations in cancer cells but not in normal cells. Indeed, oxidative stress has been shown to play an important role in the anticancer activity of several chemotherapeutic agents commonly used in cancer treatment[39−41] and in the cytotoxic activity of many natural products.[42] Figure 3C shows that preincubation of A549 cells with MnTMPyP (a superoxide dismutase mimetic that prevent oxidative stress by reducing superoxide anion levels) reduced the cytotoxic activity of HTDE, therefore suggesting that the formation of superoxide anion may play a role in the cytotoxic activity of this compound.

DNA-damaging compounds (e.g., alkylating agents, cytotoxic antibiotics, and DNA topoisomerase poisons) are the most widely used anticancer drugs. Unlike non-malignant cells, tumor cells commonly have mutations in DNA repair genes that hinder the proper repair some types of DNA damage. This may explain why specific DNA-damaging drugs can selectively kill tumor cells.[43,44] Figure 3C shows that the antioxidant MnTMPyP reduced the cytotoxic activity of HTDE, therefore suggesting that the induction of oxidative stress participates in its cytotoxicity. Since oxidative stress can cause DNA damage, we used the comet assay to test if HTDE could induce DNA damage in A549 cells. Figure 3D and E shows that A549 cells exposed to HTDE had higher levels of DNA in the tail of the comets than untreated cells, therefore indicating that this compound induces DNA damage. The levels of DNA damage (expressed as percent of DNA in the tail and as tail moment) were lower than the induced by the DNA-damaging agent camptothecin.

We next used a panel of cell lines deficient in several DNA repair pathways to test if the induction of DNA damage by HTDE participates in its cytotoxic activity. All cell lines were treated with several concentrations of HTDE for 24 h, and after a recovery period of 24 h, cell viability was estimated with the MTT assay. The cytotoxic activity of HTDE against cells deficient in homologous recombination, nonhomologous end joining, mismatch repair, and Fanconi anemia was similar than that against parental cells (results not shown), therefore indicating that the cytotoxic activity of this compound is not affected by defects in these DNA repair pathways. However, results represented in Figure 3F show that HTDE 10 μM was more cytotoxic in cells deficient in base excision repair

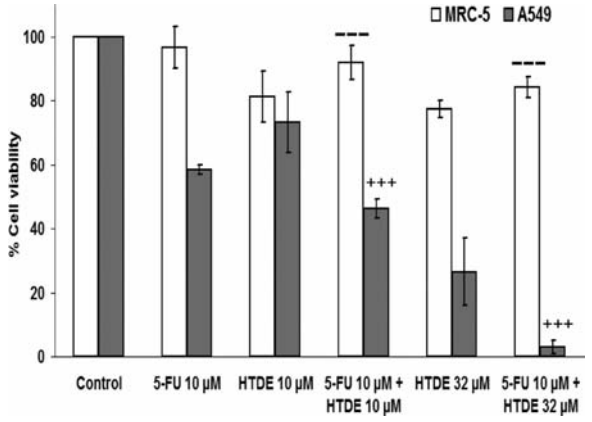

Figure 4. Cytotoxic activity of hydroxytyrosyl dodecyl ether in combination with the anticancer drug 5-fluorouracil. A549 cancer cells and MRC5 normal cells were exposed for 48 h to hydroxytyrosyl dodecyl ether (HTDE, **15**) alone and in combination with 5-fluorouracil (5-FU). The percentage of cell viability (mean ± SEM) was determined with the MTT assay, and the parameter Combination Index (CI) was calculated with the computer software Compusyn. A CI value of <1 is considered to be synergism and is represented by "+++++" for very strong synergism (CI < 0.1), "++++" for strong synergism (CI = 0.1−0.3), "+++" for synergism (CI = 0.3−0.7), "++" for moderate synergism (CI = 0.7−0.85), and "+" for slight synergism (CI = 0.85−0.9). A CI value between 0.9 and 1.1 corresponds with additive effect and is indicated with "±". A CI value of >1 is considered to be antagonism and is represented by "-" for slight antagonism (CI = 1.1−1.2), "--" for moderated antagonism (CI = 1.2−1.45), "---" for antagonism (CI = 1.45−3.3), "----" for strong antagonism (CI = 3.3−10), and "-----" for very strong antagonism (CI > 10).

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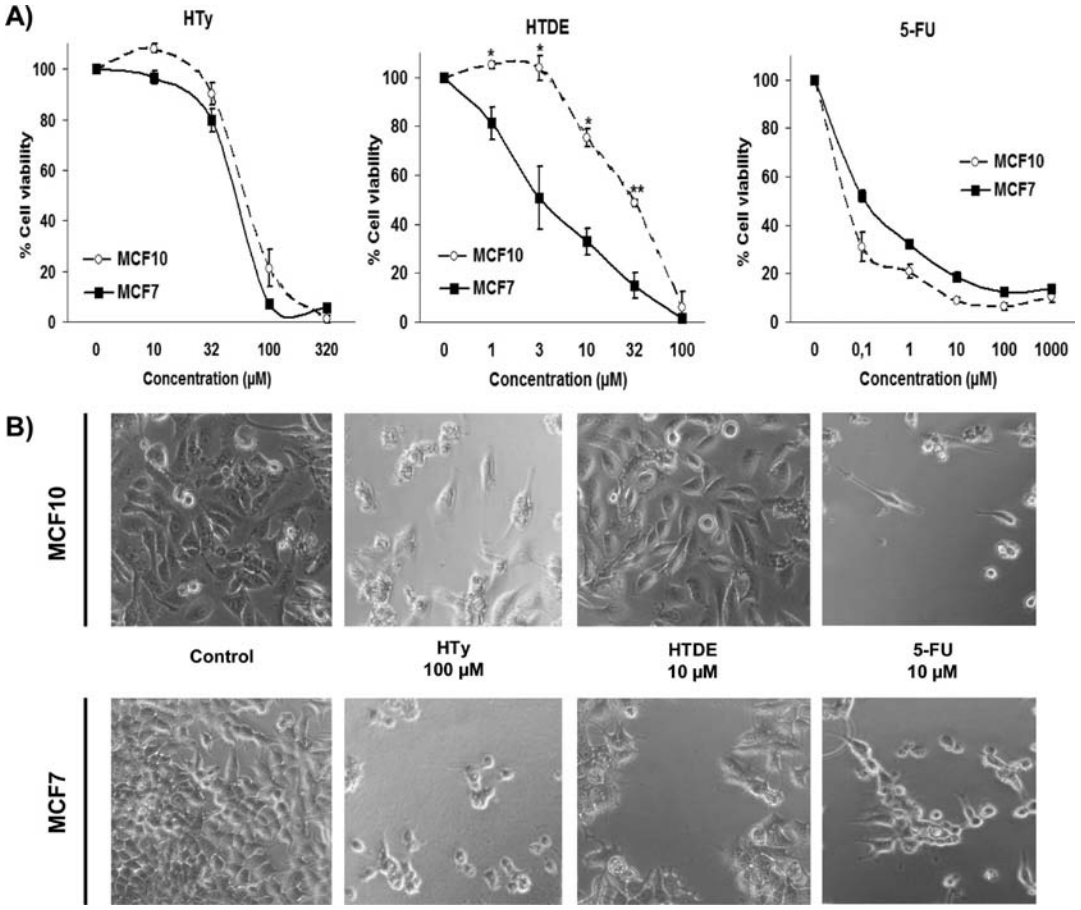

Figure 5. MCF7 breast cancer cells are more sensitive than MCF10 normal breast epithelial cells to the cytotoxic activity of hydroxytyrosyl dodecyl ether (HTDE, **15**). (A) Percentages of cell viability (mean ± SEM) in MCF10 and MCF7 cells exposed to hydroxytyrosol (HTy), HTDE, and 5-FU for 48 h (MTT assay). (B) Representative photographs of MCF10 and MCF7 cells after treatment with hydroxytyrosol (100 μM), HTDE (10 μM), and 5-FU (10 μM) for 48 h.

(EM9-V) than in parental cells (EM9-XH). HTDE was 1.6-fold more cytotoxic against EM9-V cells than on parental EM9-XH cells, with IC_{50} values (mean ± SEM; μM) of 9.09 ± 0.75 in EM9-V cells and 14.59 ± 0.64 in EM9-XH cells. The base excision repair (BER) pathway plays a crucial role in the repair of oxidative DNA damage;[45] indeed, cells deficient in BER (EM9-V) have been shown to be more sensitive to hydrogen peroxide than parental cells (EM9-XH).[46] Under our experimental conditions, hydrogen peroxide was 3,8-fold more cytotoxic in EM9-V cells than in EM9-XH cells (positive control), and hydroxytyrosol did not induce selective cytotoxicity in EM9-V cells vs EM9-XH cells (negative control). Taken as a whole, our data suggest that HTDE induces oxidative damage that may participate in its cytotoxic activity.

Since most cancer chemotherapy regimens include a combination of drugs, we assessed the cytotoxic activity of HTDE in combination with the anticancer drugs 5-FU in A549 lung cancer cells and MRC5 non-malignant lung fibroblasts. Then, we calculated the parameter Combination Index (CI) with the computer software Compusyn; this parameter is based on the Chou-Talalay method and offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations.[47] A549 and MRC5 cells were exposed for 48 h to HTDE (10 and 32 μM) with and without 5-FU 10 μM (HTDE and 5-FU were added simultaneously when tested in combination). Cell viability was then estimated with the MTT assay. Figure 4 shows that the combination of HTDE with 5-FU displayed a synergistic cytotoxicity in the A549 lung cancer cell line. Interestingly, the combination of HTDE with 5-FU induced an antagonism in the MRC5 non-malignant cell line. In other words, HTDE and 5-FU given in combination killed more cancer cells and fewer normal cells than any of these compounds given individually.

Finally, to further investigate the selective cytotoxicity of HTDE against cancer cells, MCF7 breast cancer cells and MCF10 normal breast epithelial cells were exposed to several concentrations of this compound for 48 h and cell viability was estimated with the MTT assay. The parent compound hydroxytyrosol, the anticancer drug 5-FU, and the most cytotoxic hydroxytyrosol alkyl ether derivative in A549 lung cancer cells (hydroxytyrosyl hexadecyl ether) were also tested under these experimental conditions. Only HTDE induced a clear cytotoxic selectivity; HTDE was 6.16 times more cytotoxic against MCF7 cells than against MCF10 cells. The IC_{50} values (mean ± SEM; μM) in MCF10 normal breast epithelial cells and MCF7 breast cancer were, respectively, hydroxytyrosol (64.43 ± 6.74; 50.16 ± 2.27); HTDE (29.50 ± 2.07; 4.79 ± 1.59), 5-fluorouracil (<0.1; 0.16 ± 0.03) and hydroxytyrosyl hexadecyl ether (9.79 ± 1.11; 12.00 ± 0.69). Figure 5A shows that HTDE displayed a marked selective cytotoxic activity in the 1−30 μM range and that this highly selective cytotoxicity is not shared by the parent compound hydroxytyrosol or the anticancer drug 5-FU. Figure 5B seeks to visually show changes in cell number and morphology induced by each of these compounds in both cell lines. It is

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shown that hydroxytyrosol 100 μM, HTDE 10 μM, and 5- FU 10 μM induce similar changes in MCF7 breast cancer cells (reduction of cell number and alteration of cell morphology); however, HTDE is the only compound that does not appreciably alter the number and morphology of non-malignant MCF10 cells.

Our results suggest that HTDE has cancer chemotherapeutic potential rather than chemopreventive potential. Figure 5A shows that HTDE 3 μM can inhibit the growth of MCF7 breast cancer cells without affecting the growth of MCF10 normal breast cells. It is unknown whether the oral administration of HTDE can lead to such concentrations in plasma and tissues. Previous experiments suggest that hydroxytyrosol alkyl ether derivatives are stable when digested in vitro, are rapidly absorbed, and are metabolized only partially.[27−29] Indeed, HTDE and related hydroxytyrosol alkyl ether derivatives were found to exert a neuroprotective effect in rats when administered orally, therefore suggesting that HTDE is bioavailable after oral administration. In any case, the possible low oral bioavailability and high metabolism of HTDE could be overcome with a sustained intravenous infusion. This strategy could be used to obtain and maintain cytotoxic concentrations of HTDE in plasma and tissues, which would maximize its therapeutic potential. Future animal studies are needed to evaluate the anticancer potential of HTDE in vivo.

The possible exploitation of the biological properties of hydroxytyrosol alkyl ether derivatives described in this and previous reports[25,26,30,31] would require an adequate supply of these derivatives. Several million tons per year of wastes are estimated to be produced worldwide by the olive oil industry, and interestingly, hydroxytyrosol alkyl ether derivatives can be synthesized easily and in high yield using hydroxytyrosol obtained from olive oil waste waters.[23,48]

In summary, since accumulating data suggest that the olive oil constituent hydroxytyrosol has anticancer activity, we have evaluated the selective cytotoxic activity of a series of hydroxytyrosol alkyl ether derivatives in relation to that of hydroxytyrosol and have investigated possible mechanisms involved in the cytotoxicity of the most selective compound (HTDE, **15**). Our results showed that the cytotoxic activity of HTDE was more potent and selective than that of its parent compound hydroxytyrosol.

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Notes

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