**In Vitro** Antitumor Activity of Olive Oil Tyrosol and Hydroxytyrosol and their Methyl Carbonate Derivatives

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**Abstract**

A Mediterranean diet rich in olive oil has been associated with health benefits in humans. The phenolic compounds found in virgin olive oil have demonstrated antitumor activity and antioxidant properties. The aim of this study was to confirm the *in vitro* antiproliferative effect of tyrosol (TYR) and hydroxytyrosol (HTYR) on human cancer cell line and to investigate and compare the antitumor effect of methyl carbonate derivatives of these natural compounds as tyrosol methyl carbonate (TYRMC) and hydroxytyrosol methyl carbonate (HTYRMC) synthesized in our laboratory by an eco-friendly procedure. Four human tumor cell lines of melanoma (M14), pulmonary (H125), colon (WiDr) and promyelocytic leukaemia (HL60) were exposed to the presence of 3.125, 6.25, 12.5, 25, 50 and 100 mcg/ml of TYR, HTYR, TYRMC and HTYRMC for 24, 48 and 72 h. The antiproliferative activity was assayed by counting the cells in trypan blue, and the apoptosis induction and cell cycle profiles were evaluated by flow cytometry analysis. A significant growth inhibition was observed in each tumor cell line exposed to these molecules in the experimental conditions described above. In addition, this study demonstrated that HTYRMC results more effective in cell growth inhibition and apoptosis induction reaching 98.2% at the concentration of 100 mcg/ml. However, further studies are necessary to better understand the mechanisms of these molecules on tumour cell growth.

**Keywords:** antiproliferative activity, olive oil, phenolic compounds

**Abbreviations:** HTYR, hydroxytyrosol; HTYRMC, hydroxytyrosol methyl carbonate; TYR, tyrosol; TYRMC, tyrosol methyl carbonate

**INTRODUCTION**

Natural products have long been considered as excellent sources for drug discovery given their structure diversity and wide variety of biological activities. Over the last five decades, a large number of papers have reported that natural compounds isolated from plants, including polyphenols, exhibit healthy effects on humans (Manach 2005). In the Mediterranean area, the daily consumption of wine and virgin olive oil is related to the protection against several kinds of cancer (Kontou 2011). The main responsible of these benefits are phenolic compounds such as tyrosol (TYR) and hydroxytyrosol (HTYR) (Fig. 1A) present in these food and beverage (Cornwell 2008). In virgin olive oil, they derive from the hydrolysis of secoiridoids during olive oil storage. Their concentration is related to the olive tree variety, climatic and agronomic conditions, degree of maturation at harvest, and the manufacturing process (Allouche 2007).

Currently, many studies reported the *in vitro* and *in vivo* biological activities of phenolic compounds naturally present in virgin olive oil. Among them, anti-inflammatory, cardioprotective antioxidant and chemopreventive effects in various types of cancers were claimed (Owen 2000; Visioli 2002). It has been well established that HTYR is a potent antioxidant capable to scavenge oxygen and nitrogen free radicals, to inhibit Low Density Lipoprotein (LDL) oxidation, platelet aggregation and endothelial cell activation and to protect against DNA damage. Moreover, HTYR is able to reduce the synthesis of prostaglandin E2 blocking the transcription of COX-2 and 5-lipoxygenase, thereby reducing the chronic influence associated with diseases such as cancer (Loru 2009; Rodriguez-Ramiro 2011; Bayram 2012).

More recently, the anti-proliferative and pro-apoptotic properties of HTYR on breast (MDA and MCF-7), prostate (LNCap and PC3) colon (HT-29, SW480 and HCT116) gastric (AGS) and melanoma (M14) cancer cell lines were described (D’Angelo 2005; Corona 2009; Obied 2009; Bernini 2011; Bouallagui 2011). Conversely, the *in vitro* antitumor effects of TYR are rarely described (Ahn 2008; Deiana 2010; Warleta 2011).

Studies in human, animal and cellular systems suggest that phenols from virgin olive oil are capable of inhibiting several stages in carcinogenesis, including metastasis (Hashim 2008). Unfortunately, the bioavailability of TYR and HTYR in humans is poor, and they are found in biological fluids mainly as conjugated metabolites (Khymenets...
MATERIALS AND METHODS

Chemicals and instruments

Reagents and solvents of high analytical grade were purchased from Sigma Aldrich (Milan, Italy). 2-Lodoxbenzoic acid (IBX) was prepared in laboratory as described in the literature (Frigerio 1999). Silica gel 60 F254 plates and silica gel 60 were furnished from Merck (Milan, Italy).

HPLC analyses were performed on a Varian Prostar 325 apparatus equipped with a Varian Pursuit 5 μm C18 column (150 × 4.6 mm) and a dual wavelength UV-Vis detector selected on λ = 280 nm. Elutions were carried out at a 1 ml/min flow rate using a H2O/CH3CN mixture (90:10, v/v) for the first minute and a gradient to 40:60 within the following 30 min. GC-MS analysis was performed on a Shimadzu VG 70/250S apparatus equipped with a CP-SIL 8 CB-MS column (25 m, 0.25 mm and 0.25 mm film thickness). The analyses were performed using an isothermal temperature profile of 100°C for 2 min, followed by a 10°C/min temperature gradient until 280°C for 15 min. The injector temperature was 280°C. 1H and 13C NMR spectra were recorded in CDCl3 (99.8% in deuterium) and in CD3OD (99.8% in deuterium) using a Bruker 400 MHz spectrometer.

Cell culture

Solid human tumour cell lines H125 (non-small cell lung carcinoma, obtained from American Type Culture Collection, Rockville, MD, ATCC), WIDR (colon carcinoma, obtained from ATCC) and M14 (melanoma donated by S. D’Atri IDI IRCCS, Rome, Italy) were maintained in RPMI 1640 Euroclone (H125 and M14) or DMEM Euroclone (WIDR) containing 10% fetal calf serum (Invitrogen, Irvine CA, USA) 1% l-glutamine (Invitrogen) 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma chemical co. St. Louis, MO) hereafter referred as Complete Medium (CM).

Human promyelocytic leukemic cell line (HL-60 obtained from ATCC) employed for the present study was cultured in CM containing RPMI 1640 Medium Euroclone. All cell lines were maintained in 5% CO2 in air humidified at 37°C.

Cells were routinely sub-cultured and cells in exponential growth phase were used for all experiments. Cells that were grown as monolayers were detached from cultures by trypsinization. Cells were washed twice with PBS and treated with 0.25% trypsin-EDTA (Invitrogen) for 10 min at 37°C in 5% CO2 humidified atmosphere. The trypsinization effect was neutralized with CM. The cells were collected after centrifugation at 1000 rpm for 5 min and washed with PBS for viability assays.

Drug treatment and cell proliferation assay

Aliquots of TYR, HTRY, TYRMC and HTYRMC were stored at −20°C in ethanol (EtOH). The compounds were thawed just before use and diluted in RPMI 1640 to desired concentrations. For the inhibition of tumour cell proliferation experiments, the tumour cells H125, WIDR, H125 and HL60 were placed in a 24-well (Falcon Becton Dickinson) tissue culture plate (1 × 104 cells/well) and incubated 3 h at 37°C in 5% CO2 in CM to permit the adherence of cell growing in monolayer. Then the polyphenols were added to cultures at the concentration of 3.125, 6.25, 12.5, 25, 50 and 100 mcg/ml. Controls were performed by adjusting the respective EtOH concentration to that of the treated samples. Cells with only RPMI medium were included as controls. All cells were treated with the compounds at 37°C in 5% CO2 for 24, 48 and 72 h.

Cell proliferation, measured as the cellular growth of treated cells vs. untreated controls, was performed after 24, 48 and 72 h of treatment. After trypsinization, the cells were harvested and counted in the presence of Trypan Blue in a Burker chamber. Each experiment was repeated three independent times.

Cell sensitivity to TYR, HTRY, TYRMC and HTYRMC treatment was expressed in terms of IC50 (i.e. the compound concentration producing 50% of inhibition cell growth, calculated on the regression line in which the number cells values were plotted against the logarithm of drug concentration).

Cell cycle assay and apoptosis

Tumor cells were seeded in 24-well culture plates at 1 × 105 cells/well and treated with different doses of TYR, HTRY, TYRMC and HTYRMC for 24, 48 and 72 h.

After incubation, 1 × 105 cells/ml were washed in cold PBS, fixed with cold 70% ethanol and stored at −20°C for 24 h. At least 1 × 10⁵ events per sample were acquired and analyzed on an FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA USA) after propidium iodide labeling (PI, Sigma) and 100 KU/ml of RNase A (Sigma) exposure. The percent coefficient of variation (CV) relative to G2 peak distribution was around 3%. The percentage of cells in G0/G1, S and G2/M phases were calculated using ModFitLT software (Becton-Dickinson). Each experiment was repeated three independent times.

Flow cytometry was also used to detect apoptosis in non-treated or TYR, HTRY, TYRMC and HTYRMC treated cells for 24, 48 and 72 h. Cells were then washed in PBS and fixed with 50% acetone/methanol (1:4) in 50% PBS at 4°C for 18 h. After two washes in PBS, the cells were suspended in PBS (1 × 10⁶ cells/ml) containing 50 μg/ml of propidium iodide (PI, Sigma) and 100 KU/ml of RNase A (Sigma) and incubated in the dark at room temperature for 30 min. The DNA content per cell was evaluated by PI fluorescence measured on a linear scale using the FACScan flow cytometer (Becton-Dickinson, San Jose, CA USA) with an argon ion laser emitting at 488 nm. Data collection of 10,000 cells/sample was gated utilizing forward light scatter and side light scatter dot plot to exclude cell debris and aggregates. Apoptotic cells were identified as the sub-G1 hypochromic peak in DNA staining profiles. All data were stored and analysed by using the Cell Quest Software.

Statistical analysis

All test were run in triplicate for each experimental condition and each experiment was repeated at least 3 times; the results are reported as means ± SD. Data were analyzed by a one-way ANOVA with a post hoc Tukey’s test.

RESULTS

In vitro antiproliferative activity on the human cancer cell lines

Human M14 melanoma cell line and human promyelocytic leukemic cell line HL60 were tested for sensitivity to TYR, HTRY, TYRMC and HTYRMC.

For this purpose, M14 and HL60 cells were incubated with different doses of these compounds at concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 mcg/ml, for 24, 48 and 72 h. Cell viability was assessed by trypan blue dye exclusion assay. The results show that all polyphenols induced a time and dose dependent inhibition of M14 cell growth. However, the rate of growth inhibitory effect changed among the different compounds when compared at the same doses (data not shown). After 72 h of treatment the cytotoxic effects significantly increased with all drugs used. The percentages of cell growth inhibition, as illustrated in Table I show that, respect to other compounds HTYRMC induced a markedly significantly inhibitory activity at all doses examined reaching 98.2% at a concentration of 100 mcg/ml. Conversely, excluding the dose of 100 mcg/ml, no statis-
In order to further investigate the mechanism underlying the anti-proliferative activity of HTYR and HTYRMC, the cell cycle distribution has been analysed by flow cytometry. The cell cycle progression was assessed following treatment with 100 mcg/ml of both compounds for 24 h.

Table 1 Percent growth inhibition of M14 melanoma cell line treated with TYR, HTYR, TYRMC and HTYRMC for 72 h of culture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TYR</th>
<th>TYRMC</th>
<th>HTYR</th>
<th>HTYRMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 mcg/ml</td>
<td>31.2</td>
<td>38.1</td>
<td>22.4</td>
<td>41.8</td>
</tr>
<tr>
<td>25 mcg/ml</td>
<td>40.3</td>
<td>41</td>
<td>31.4</td>
<td>65.9</td>
</tr>
<tr>
<td>50 mcg/ml</td>
<td>41.9</td>
<td>47.5</td>
<td>41.2</td>
<td>87.2</td>
</tr>
<tr>
<td>100 mcg/ml</td>
<td>55.1</td>
<td>77</td>
<td>81.2</td>
<td>98.2</td>
</tr>
</tbody>
</table>

Effect of TYR, HTYR, TYRMC, and HTYRMC on tumor cell growth of M14 melanoma cell line. Cells were cultured in CM or CM containing the indicated concentrations of TYR, HTYR, TYRMC, and HTYRMC for 72 h. Control group were treated with EtOH alone. Cell proliferation was determined by the trypan blue dye exclusion assay. Measurements were done in triplicate. Data are expressed in terms of percent growth inhibition of M14 treated cell line respect to the control. Values were calculated by the mean of three separate experiments.

Table 2 Percent growth inhibition of HL60 cell line treated with TYR, HTYR, TYRMC and HTYRMC.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TYR</th>
<th>TYRMC</th>
<th>HTYR</th>
<th>HTYRMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mcg/ml</td>
<td>3.3</td>
<td>15.4</td>
<td>2.7</td>
<td>7.9</td>
</tr>
<tr>
<td>50 mcg/ml</td>
<td>9.7</td>
<td>31.9</td>
<td>10.9</td>
<td>19.6</td>
</tr>
<tr>
<td>100 mcg/ml</td>
<td>47.5</td>
<td>46.1</td>
<td>34.2</td>
<td>59.6</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mcg/ml</td>
<td>3.8</td>
<td>7.5</td>
<td>9.8</td>
<td>13.9</td>
</tr>
<tr>
<td>50 mcg/ml</td>
<td>23.7</td>
<td>20.7</td>
<td>17.1</td>
<td>42.5</td>
</tr>
<tr>
<td>100 mcg/ml</td>
<td>41.6</td>
<td>48.4</td>
<td>66.8</td>
<td>79.5</td>
</tr>
<tr>
<td>72 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mcg/ml</td>
<td>3.4</td>
<td>12.3</td>
<td>12.7</td>
<td>18.6</td>
</tr>
<tr>
<td>50 mcg/ml</td>
<td>13.9</td>
<td>39.8</td>
<td>14.9</td>
<td>35.8</td>
</tr>
<tr>
<td>100 mcg/ml</td>
<td>48.9</td>
<td>55.2</td>
<td>78.3</td>
<td>89.1</td>
</tr>
</tbody>
</table>

Effect of TYR, HTYR, TYRMC, and HTYRMC on tumor cell growth of HL60 cell line. Cells were cultured in CM or CM containing the indicated concentrations of TYR, HTYR, TYRMC, and HTYRMC for 24, 48 and 72 h. Control group were treated with EtOH alone. Cell proliferation was determined by the trypan blue dye exclusion assay. Measurements were done in triplicate. Data are expressed in terms of percent growth inhibition of HL60 treated cell respect to the control. Values were calculated by the mean of three separate experiments.

Table 3 IC50 values of TYR, TYRMC, HTYR, HTYRMC.

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>TYR</th>
<th>TYRMC</th>
<th>HTYR</th>
<th>HTYRMC</th>
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<tbody>
<tr>
<td>M14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>69.6</td>
<td>42</td>
<td>76.7</td>
<td>223.9</td>
</tr>
<tr>
<td>48 h</td>
<td>41.7</td>
<td>48.5</td>
<td>42.2</td>
<td>78.9</td>
</tr>
<tr>
<td>HL125</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>55.9*</td>
<td>39.8</td>
<td>46.3*</td>
<td>87.7**</td>
</tr>
<tr>
<td>48 h</td>
<td>9.9**</td>
<td>3.4**</td>
<td>39.3</td>
<td>55.2**</td>
</tr>
<tr>
<td>WIDR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HL60</td>
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Discussion

Epidemiological and prospective studies have illustrated the beneficial effects of bioactive compounds that naturally occur in many vegetable foods of the Mediterranean diet (Patil 2009). In particular, some of these studies have shown an inverse correlation between olive oil consumption and incidence of various types of cancers e.g. breast (Trichopoulos 1995), prostate (Hodge 2004), oral cavity (Franceschi 1999), and colon (Braga 1999). Recently, it was hypothesized that the cancer preventive capacity of olive oil could be mediated by the presence of potent antioxidant phenolic compounds that were shown to possess several biological activities (Raedderstorff 2009).

Among the different phenols known to be present in olive oil, TYR and HTYR have received particular attention because of their association with the inhibition of both initiation and promotion in carcinogenesis (Cornwell 2008; Hashim 2009). However, they have been reported to have a poor bioavailability in humans since they are found in biological fluids mainly as conjugated metabolites (Khymenets et al. 2009). In the present study we investigated the antitumor activity of two additional derivatives of TYR and HTYR such as TYRMC and HTYRMC synthesized in our laboratory. These compounds are more lipophilic than TYR and HTYR showing the alcoholic group derivatized by a carboxylate moiety. To evaluate and compare the antitumor activity of these four polyphenols, a human melanoma cell
A human promyelocitic leukemia cell line (M14) and a human promyelocytic leukemia cell line (HL-60) were treated for 24, 48 and 72 h with increasing amounts of TYR, HTYR, TYRMC and HTYRMC. The results showed that the growth inhibition increased with concentration and time of exposure to every one polyphenols tested as illustrated in Table 1 and Table 2. After 72 h of exposure the cell growth inhibition reaches more than 60-90%, in particular the HTYRMC exposure showed the most antiproliferative effect. Comparison of the inhibitory effect of HTYRMC respect to HTYR on four tumor cell line (Table 3) showed that after 48 h of treatment the IC_{50} of HTYRMC in M14, H125 and HL60 are significantly (\(P < 0.01\)) lower respect to the IC_{50} of HTYR. Moreover it is interesting to observe that the IC_{50} value is strictly dependent on the cell type used.

Additionally, we observed significant morphological changes in all tumor cells treated with HTYRMC. Cell shrinkage and formation of apoptotic bodies were clearly observed suggesting the cell growth arrest could be related to apoptosis induction (data not shown).

Previous studies demonstrated that HTYR causes growth arrest and apoptosis in various cell line among which M14 (D'Angelo 2005) and HL60 cell lines (Fabiani 2009). Our results (Fig. 3) confirm the proapoptotic effect of HTYR and show a significative percent increase of the number cells in apoptosis evaluated in tumor lines treated with HTYRMC for 72 h. Particularly, the percent of increase of number cells in apoptosis depends on the tumor line examined.

Recently published work suggests that the anti-proliferative and pro-apoptotic properties of HTYR on HL60 cells may be mediated by the accumulation of hydrogen peroxide (\(\text{H}_2\text{O}_2\)) in the culture medium (Fabiani 2009). Fabiani (2011) suggest that the culture medium components interfere with \(\text{H}_2\text{O}_2\)-produced by HTYR that are the basis of the proapoptotic mechanism. In our experiments, the cell lines were grown and treated with polyphenols in different culture medium, this could explain the different level of

![Fig. 2 Effect of treatment with 100 mcg/ml of HTYR or HTYRMC on cell cycle phase distribution of M14, WiDR, H125 and HL60 cell lines. The cells were cultured in CM containing the indicated concentration of HTYR or HTYRMC for 24 h, harvested, stained with PI and processed for flow cytometric analysis of DNA content. Control groups were treated with EtOH alone. Data are representative from at least three independent experiments. The results illustrated in the figure have been confirmed by further similar experiments. The increase of the percentage of cells in G1 cell cycle phase reached a statistical (\(P < 0.05\)) as shown by student’s t-test.](image-url)
apoptosis that we founded.

It is reasonable to hypothesize that the differences in the level of apoptosis induction in different cell lines may depend on different mechanisms involved. HTYR interferes with several signalling pathways that control proliferation and apoptosis, among these the FAS inhibition is related to cell line used (Notarnicola 2011).

The inhibition of cell cycle progression by HTYR has been previously described (Rafehi H 2012). The results obtained in our experiment showed that in all line used the treatment with HTYR exhibited statistically significant block of G_{0}/G_{1} phase transition manifested by the increase of cell number in G_{0}/G_{1} phase. Our results show that only in WiDR and M14 cell line HTYRMC induced a significantly stronger block of the cell cycle at the G_{0}/G_{1} phase respect to those obtained with HTYR.

These results are in agreement with previous results that show that HTYR inhibits the proliferation of HL60 human promyelocytic leukemia cells and alters the cell cycle progression, inducing an accumulation of cells in the G_{2}/M phase (Fabiani 2002). In contrast, previous studies have been found that HTYR induces growth arrest and apoptosis in human colon carcinoma HT-29 cells and the growth arrest was associated with the accumulation of cells in S and G_{2}/M phases of the cell cycle (Guichard 2006). The molecular mechanisms by which HTYR interferes with the cell cycle and induces apoptosis on tumour cells are not known; however, it is well known that HTYR is able to modify the expression of important proteins involved in the regulation of these processes. The cell cycle is regulated by the sequential expression of cyclins, which activate various cyclin-dependent kinases (CDK) and by the selective modulation of the expression of important proteins involved in the regulation of these processes.

In this study we have confirmed that most important phenolic compounds of olive oil show anticancer activity by antiproliferative and proapoptotic activity and that this is detectable in four different cell lines. Our findings may also support the hypothesis that novel compounds might exert a more antitumour activity than the natural compounds by inducing a greater number of cells in cell cycle arrest and apoptosis. This is particularly evident in the case of HTYRMC suggesting a marked and antitumor effect of this synthetic compound.

CONCLUSIONS

This study showed that the phenolic compounds TYR, HTYR, TYRMC and HTYRMC exert a significant antiproliferative effect in different tumor cell lines in vitro. Particularly the synthesized methyl carbonate derivatives had the most antitumor activity. However, the subcellular events contributing to HTYR and HTYRMC antiproliferative activity on tumor cells remain completely unknown therefore, further studies are necessary to explore molecular mechanisms of tumor suppressive effect of these derived compounds as well as other olive oil products.

REFERENCES


tive and efficient carboxymethylation of the alcoholic chain of phenols by dimethyl carbonate (DMC). *Tetrahedron Letters* 63, 7000-7003

Bouallagui Z, Han J, Isoda H, Sayadi S (2011) Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food and Chemical Toxicology* 49, 179-184


