



Original Contribution

Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells

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Abstract

Previous studies showed that long-wave ultraviolet (UVA) radiation induces severe skin damage through the generation of reactive oxygen species and the depletion of endogenous antioxidant systems. Recent results from our laboratory indicate a dramatic increase of both lipid peroxidation products (TBARS) and abnormal L-isoaspartyl residues, marker of protein damage, in UVA-irradiated human melanoma cells. In this study, the effects of hydroxytyrosol (DOPET), the major antioxidant compound present in olive oil, on UVA-induced cell damages, have been investigated, using a human melanoma cell line (M14) as a model system. In UVA-irradiated M14 cells, a protective effect of DOPET in preventing the uprise of typical markers of oxidative stress, such as TBARS and 2',7'-dichlorofluorescein (DCF) fluorescence intensity, was observed. In addition, DOPET prevents the increase of altered L-isoAsp residues induced by UVA irradiation. These protective effects are dose dependent, reaching the maximum at 400 μ M DOPET. At higher concentrations, DOPET causes an arrest of M14 cell proliferation and acts as a proapoptotic stimulus by activating caspase-3 activity. In the investigated model system, DOPET is quantitatively converted into its methylated derivative, endowed with a radical scavenging ability comparable to that of its parent compound. These findings are in line with the hypothesis that the oxidative stress plays a major role in mediating the UVA-induced protein damage. Results suggest that DOPET may exerts differential effects on melanoma cells according to the dose employed and this must always be taken into account when olive oil-derived large consumer products, including cosmetics and functional foods, are employed.

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Introduction

The peculiar climate of the Mediterranean basin, characterized by warm and prolonged sunlight irradiation, favors development of plants, such as olive trees and grape, whose fruits require a high proportion of antioxidant molecules [1]. The synthesis of pigments such as flavonoids, anthocyanins, and polyphenols, activated by sun irradiation [2,3], results in dark-colored fruits that, by this way, protect themselves from the noxious effects of prolonged exposure to sunlight. A Mediterranean diet, rich in fruits and vegetables, grants an elevated intake of these antioxidants that may contribute to its beneficial effects on human health. A number of epidemio-

Abbreviations: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; COMT, catechol-O-methyltransferase; DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DOPET, 3,4-dihydroxyphenylethanol; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; MOPET, 4-hydroxy-3-methoxyphenyl-ethanol; HVA, homovanillic alcohol; PBS, phosphate-buffered saline; PIMT, protein L-isoaspartate(D-aspartate)-O-methyltransferase (EC 2.1.1.77); ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive lipid peroxidation end products; SE, standard error; UVA, long-wave ultraviolet; UVB, medium-wave ultraviolet.

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logical studies, indeed, indicate that these dietetic habits may lower the incidence of several degenerative pathologies, including coronary heart diseases and cancer [4–6].

A case-control study in Australia suggests the existence of an inverse relationship between the risk of skin cancer and the high intake of antioxidant-containing foods [7]. In this respect, it has been amply reported that natural antioxidants can exert a protective effect against skin inflammation and cancer, induced by ultraviolet (UV) radiation [8]. Medium-wave (UVB)¹-induced carcinogenesis in mouse was suppressed when a green tea polyphenolic fraction was topically applied to the skin or orally administered in the drinking water [9,10]. It has been shown that quercetin, intraperitoneally administered to rats before long-wave UV (UVA) irradiation, decreases significantly the malondialdehyde concentration and slows down the decrease of enzymatic antioxidant activities [11]. Moreover, a diet rich in lycopene protects the skin against UV-induced erythema formation in humans [12]. Recently, F'guyer et al. [13] demonstrated that topical application of resveratrol to hairless mice results in a significant inhibition of UV-induced skin edema. Recent reports indicate that both orally administered and topically applied vitamin E prevents the UVB-induced skin carcinogenesis in mice [14]. The effect of several antioxidants has been tested in UVA-irradiated human skin fibroblasts, suggesting that vitamin E and vitamin C are potential photoprotectors [15]. Finally, in the recent years it has been reported that olive oil application exerts a protective effect against UVB-induced murine skin tumors [16]. These data encouraged the use of olive oil-based sun lotions, containing high levels of antioxidant compounds.

Hydroxytyrosol (3,4-dihydroxyphenylethanol; DOPET) is the main *ortho*-diphenolic compound found in olive oil and responsible for its antioxidant properties [17,18]. In addition, it should be noted that DOPET, in addition of being an olive oil component, is also a dopamine metabolite [19,20]. DOPET has been shown to function as an efficient scavenger of peroxy radicals [21] and contributes toward determining the shelf-life of the oil, preventing its auto-oxidation [17,18]. Nevertheless the possibility that protection exerted by DOPET may involve actual prevention of radical generation cannot be ruled out. The biological activities of DOPET have been explored by several groups as reviewed by Manna et al. [22]. DOPET in vitro prevents LDL oxidation [23] and platelet aggregation [24] and inhibits 5- and 12-lipoxygenases [25]. It also exerts an inhibitory effect on peroxynitrite-dependent DNA base modifications and tyrosine nitration [26]. Experiments from our laboratory have demonstrated that DOPET, which effectively permeates cell membranes via a passive diffusion mechanism [27], counteracts the cytotoxic effects of reactive oxygen species (ROS) in various human systems. Preincubation of intestinal Caco-2 cells with DOPET prevents the typical damages of oxidative stress [28]. Similarly, DOPET exerts a protective effect

against the H₂O₂-induced oxidative hemolysis and malondialdehyde formation in red blood cells [29]. Moreover, this diphenol exerts an antiproliferative effect, inducing apoptosis in HL-60 cells as well as in resting and activated peripheral blood lymphocytes [30]. DOPET bioavailability and metabolism have been widely investigated: it is interesting to note in this respect that the molecule, when intravenously injected to rats, rapidly distributes in all organs and tissues, where it undergoes extensive metabolic transformation [20].

Cultured melanoma cells are a suitable model system for studying the mechanisms by which UVA irradiation might induce structural and functional alterations at the tissue level, as well as the biological implications of such modifications [31–33]. Nevertheless previous studies suggest that melanoma cells may behave slightly differently from normal melanocytes in their antioxidant response to oxygen [34]; thus suggesting careful extrapolation of results to situations which are operative in vivo. A number of reports, so far, contributed to establish the role of ROS in the mechanism(s) of cell damages caused by UV radiation [16,35]. Recently, a dramatic increase of both lipid peroxidation products (TBARS) and abnormal isoaspartyl residues have been reported in a human melanoma cell line (M14) exposed to increasing doses of UVA [31]. These abnormal residues, arising from Asn deamidation and/or Asp isomerization, are selectively recognized and methyl-esterified, by protein L-isoaspartate(D-aspartate)-O-methyltransferase (PIMT; EC 2.1.1.77), a ubiquitous housekeeping enzyme identified in both prokaryotes and eukaryotes [36–39]. PIMT catalyzes the transfer of a methyl group from *S*-adenosylmethionine (AdoMet) to free α -carboxyl groups of L-isoAsp residues, thus activating their conversion into normal L-aspartyl residues [40,41]. Therefore this repair reaction prevents the accumulation of potentially dysfunctional proteins in cells and tissues [40,42,43]. In addition, PIMT-mediated methyl ester formation can be used as a marker reaction to tag these abnormal residues in natural and recombinant proteins [44] and to monitor their increase associated with cell stress conditions [45–47].

Using this tool, we were able to show that in M14 cells the occurrence of L-isoaspartyl residues is an early event upon UVA-induced cell injury. A potential role of the oxidative stress in the mechanism of this UVA-induced protein damage has been hypothesized [31,48–50]. To further assess the existence of a close relationship between oxidation phenomena and protein damage, the possible effects of the natural antioxidant DOPET in this model system have been evaluated. Results indicate that hydroxytyrosol is able to prevent the increase in L-isoaspartyl residues, markers of protein damage, in M14 cells exposed to UVA irradiation. DOPET's protective effect is paralleled by a decrease of ROS production, thus confirming the role of the oxidative stress in the mechanism of UVA-induced protein damages.

Materials and methods

Chemicals

3,4-Dihydroxyphenyl[ethanol- ^{14}C] (2.25 mCi/mmol) was custom-synthesized by NEN Life Science Products (Cologno Monzese, Italy). *S*-Adenosyl-L-[methyl- ^{14}C]methionine (specific activity 58 mCi/mmol) and L-[methyl- ^3H]methionine (specific activity 79 Ci/mmol) were from Amersham International (UK). Roswell Park Memorial Institute medium (RPMI), fetal calf serum (FCS), L-glutamine, penicillin-streptomycin, trypsin, phosphate-buffered saline (PBS) were purchased from Gibco Life Science Technology (S. Giuliano Milanese, MI, Italy). Adenosine, cycloleucine, 2'-7'-dichlorofluorescein diacetate (DCFH-DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA, homovanillic acid), 4-hydroxy-3-methoxyphenyl-ethanol (MOPET, homovanillic alcohol), L-homocysteine thiolactone, propidium iodide, RNase, *S*-adenosylmethionine, and *S*-adenosylhomocysteine were from Sigma.

DOPET was synthesized as described in [20]. Human recombinant PIMT isoenzyme II [51] was kind gift of Dr. Steven Clarke from the Molecular Biology Institute, University of California, Los Angeles, California.

M14 culture cell

Human melanoma M14 cells were grown as monolayers in RPMI medium with 10% (v/v) fetal calf serum containing 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in 5% CO_2 in humidified air.

For experiments, cells were detached from stock cultures by trypsinization and washed once by centrifugation; subculture was seeded in 30-mm dishes at a density of 0.8×10^6 cell/dish. Cultures were examined under an inverted light microscope on a regular basis to monitor growth and contamination [31].

Cell viability

M14 cell viability was determined using both trypan blue (0.2% w/v) exclusion test and MTT methods.

For trypan blue assay, M14 cells, after 3 days of culture, were incubated overnight (18 h) in the presence of increasing concentrations of DOPET in serum-free RPMI. After overnight incubation, cell samples were used for analysis.

MTT assay depends on the extent to which viable cells convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide to an insoluble colored formazan product that can be determined spectrophotometrically. Cells were seeded into 6-well plates at a density of 8×10^5 cell/well, grown in complete medium for 3–4 days, and washed in PBS; serum-free medium \pm DOPET was then added and incubated for different times. At the end of the incubation the medium was removed, cells were washed in PBS, and

200 μl RPMI without phenol red, containing 5 mg/ml MTT, was added to each well. Three hours later, the RPMI-MTT solution was removed and the converted dye solubilized with acid isopropanol (0.1 N HCl in absolute isopropanol). The absorbance of the resulting solution derived from each well was measured at a wavelength of 570 nm with background subtraction at 630 nm.

UVA irradiation

M14 cells, after 3 days of culture, were incubated overnight with different concentrations of DOPET in presence of serum-free RPMI. At the end of the incubation, the medium was removed, and the cells were bathed in serum/phenol red-free RPMI medium and then exposed to UVA dose [31]. UVA irradiation was performed using UVA a source (Cole-Parmer 9815-series lamps, 6 W; Cole-Parmer Instruments Co., Chicago, IL), with continuous emission spectrum with a peak at 365 nm, using a dose of 7.5 J/cm^2 (about 4 h exposure time). Light intensity was measured by means of a 365-wave ultraviolet radiometer (Cole-Parmer International Co.). In order to exclude that DOPET or MOPET may act as UVA absorbers, we made comparisons of their UV spectra with the emission spectrum of the source used in these experiments.

Determination of reactive oxygen species (ROS)

To measure the rate of ROS production and the scavenging effect of DOPET, the dichlorofluorescein (DCF) assay was performed [52].

M14 cells were incubated overnight with different concentrations of DOPET and immediately before irradiation were treated with the nonpolar, nonfluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The compound undergoes deacetylation by cytosolic esterases to form the polar, nonfluorescent dichlorodihydrofluorescein (DCFH) which, reacting with ROS, gives rise to the fluorescent derivative dichlorofluorescein. A stock solution of DCFH-DA (3.34 mM) was prepared in absolute ethanol and stored at -20°C . After 30 min at 37°C, M14 cells were irradiated with UVA (7.5 J/cm^2). Then, the medium was discarded and the cells were washed twice with prewarmed PBS and gently removed by scraping into 2 ml of PBS and kept on ice. The cellular suspension was then transferred into fluorescence cuvettes and the fluorescence intensity of the probe (λ_{exc} 502 nm; λ_{em} 520 nm; bandwidths 5 nm) was recorded. The results were expressed as fluorescent intensity per microgram protein.

TBARS assay for lipid peroxidation

The thiobarbituric acid reactive lipid peroxidation end products (TBARS) were determined on aliquots of cell culture medium, in that after UVA irradiation most of the TBARS are released by the cells.

TBARS were measured by modification of the method of the Buege and Aust [53]. Briefly, to 250 μ l of cell supernatant 500 μ l of 30% trichloroacetic acid was added, vortexed, and centrifuged at 5000g for 15 min. A 500- μ l aliquot of the acid-soluble supernatant was added to 500 μ l of 1% (w/v) thiobarbituric acid in 0.05 N NaOH and heated in a boiling-water bath for 10 min. The absorbance of the developed pink chromophore was determined at 532 nm.

In vitro assay for PIMT activity in cell lysate

Cell lysates were prepared by freezing and thawing cells at a concentration of 0.8×10^6 cells/ml in 10 mM sodium phosphate, pH 7.2, a buffer containing 1 mM EDTA, 100 mM NaCl, 0.2% Triton X-100 at 4°C. The homogenate was centrifuged for 15 min at 10,000g at 4°C [42]. The supernatant was collected and PCMT activity was determined, *in vitro*, as previously described [54].

Cytosolic extracts (30 μ g proteins) were incubated, at 37°C for 30 min, with 1.6 mg of ovalbumin (as a standard methyl-accepting substrate) in 0.1 M sodium citrate at pH 6.0, containing 30 μ M *S*-adenosyl-L-[methyl-¹⁴C]methionine in 40 μ l final volume [47]. The reaction was stopped by adding an equal volume of 0.2 M NaOH, 1% SDS. Radioactivity due to methyl incorporation was determined as previously described [55]. PIMT specific activity is expressed as U (pmol methyl ester formed \times min⁻¹ \times mg protein⁻¹). Protein concentration was estimated according to the method of Bradford [56], by means of the Bio-Rad protein assay kit.

Protein carboxyl methyl esterification assay in intact M14 cells (in situ methylation assay)

Carboxyl methylation rate was evaluated as previously described [42], with some modifications. Briefly, after overnight incubation with DOPET, M14 cells were bathed in serum-phenol red-free RPMI medium without L-methionine and irradiated with 7.5 J/cm²-dose of UVA. Cells were then washed and plated in fresh culture medium containing 20 μ M [methyl-³H]methionine, the *in vivo* precursor of *S*-adenosylmethionine and 160 μ M cycloheximide, as a protein synthesis inhibitor. In some experiments, 5 mM adenosine, 5 mM L-homocysteine thiolactone, and 25 mM cycloleucine (as protein methylation reaction inhibitors) were added to the medium [57]. After overnight incubation, the cultures were washed with PBS and the cells were lysed and kept on ice.

To analyze total methyl ester formation, 50 μ l of cell lysates was added to an equal volume of 0.1 N NaOH and incubated for 10 min at room temperature to hydrolyze protein methyl esters. The mixture was transferred to a filter paper wedged in the neck of a scintillation vial and volatile [³H] methanol was quantitated by the vapour-phase equilibration assay [58]. Background was evaluated by treating parallel samples in the presence of methylation

inhibitors. The relevant radioactivity was subtracted from total methyl incorporation values.

AdoMet specific activity was determined by HPLC analysis of the perchloric acid-soluble fraction of cytosolic extracts, evaluating the radioactivity associated with [methyl-³H]AdoMet.

Determination of S-adenosylmethionine and S-adenosylhomocysteine M14 cell content

Intracellular concentrations of *S*-adenosylmethionine (AdoMet) and *S*-adenosylhomocysteine (AdoHcy) were determined by HPLC in a perchloric acid-soluble fraction of M14 cell cytosolic extracts [59]. All samples were filtered through a 0.2- μ m pore filter before injection onto a Zorbax C8 reverse-phase column (25 cm \times 4 mm; Du Pont-New England Nuclear, Boston, MA), equilibrated with buffer A (50 mM NaH₂PO₄/10 mM heptanesulfonic acid buffered, pH 3.2), containing 4% (w/v) acetonitrile. Nucleosides were eluted with a 15-min linear gradient of 4–20% acetonitrile, followed by a 10-min linear gradient of 20–25% acetonitrile, at a flow rate of 1 ml \times min⁻¹.

In vitro evaluation of altered aspartyl residues

To quantitatively evaluate L-isoaspartyl and D-aspartyl methyl-accepting sites in cellular proteins, M14 cells were treated as above described and immediately lysed. Twenty microliters of cellular homogenate were incubated with 28 U of recombinant PCMT in 0.1 M sodium citrate (pH 6.0) containing 30 μ M *S*-adenosyl L-[methyl-¹⁴C]methionine in a 40 μ l volume at 37°C for 30 min. After alkaline hydrolysis, [¹⁴C]methanol production was measured as described above. Results are expressed as picomoles methyl ester formed \times per milligram cellular protein [31].

DOPET metabolism analysis

In order to identify DOPET's metabolites, M14 cells were incubated overnight with RPM medium containing 200 μ M [¹⁴C] DOPET (3.2×10^6 dpm). At the end of the incubation 10% TCA was added to the samples (1:1 v/v). After centrifugation at 13,000g for 15 min, the supernatant was used for HPLC analysis. HPLC separation of DOPET and its metabolites was performed by reversed-phase chromatography on 150 \times 4.6-mm C₁₈ 5- μ m column (Kromasil), using a Beckman Apparatus (Gold-126) equipped with an UV detector fixed at 278 nm. The column was eluted at a flow rate of 1.0 ml/min with acetic acid 0.2%, pH 3.1, (A)/methanol (B) as the mobile phase; the gradient was changed as follow: 95% A/5% B for 2 min, 75% A/25% B in 22 min, 0% A/100% B in 5 min, 0% A/100% B for 5 min, and 95% A/5% B in 5 min. Labeled species were identified on the basis of the retention times of authentic standard references (DOPET, MOPET, HVA, and DOPAC) [30].

Detection of caspase-3 activity

Caspase-3 activity was measured by using a CPP32/caspase-3 fluorometric assay kit (FluorAce apopain assay kit, Bio-Rad) according to the manufacturer's instructions.

Briefly, M14 cells were incubated overnight with different concentrations of DOPET and irradiated with UVA rays. For analysis of caspase-3 activity, the cells were washed with PBS twice and suspended in 150 μ l of Apopain Lysis Buffer (250 mM Hepes, pH 7.4, 50 mM EDTA, 2.5% CHAPS, 125 mM DTT). The samples were frozen and thawed five times and cells lysates were centrifuged at 18,000g for 30 min. Ten microliters of z-DEVD-AFC substrate was added to 1 ml of solution containing 20 μ g of supernatant cellular protein and incubated at 37°C for 3 h. The caspase-3-mediated cleaved AFC fluorescence signal was measured using 375 nm excitation and 530 nm emission.

Fluorescence-activated cell sorter (FACS) analysis

M14 cells were incubated overnight with different concentrations of DOPET and irradiated with UVA rays. Then, cells were harvested by trypsin digestion and washed with cold PBS. After centrifugation, cells were resuspended in PBS containing 200 μ g/ml RNase A and 15 μ g/ml propidium iodide. After incubation for 30 min at room temperature, samples were subjected to FACS analysis (BD FACS Calibur).

Statistical analysis

All results are presented as the means \pm SE.

Results and discussion

DOPET toxicity and metabolism in human melanoma cells

In order to evaluate the toxicity of DOPET on the investigated system, M14 cells were incubated overnight (18 h) with increasing amounts of this molecule and cell viability was evaluated by trypan blue exclusion method. These results indicated that 100 μ M DOPET was devoid of any significant negative effect on cell viability (Fig. 1). At a concentration as high as 600 μ M DOPET, cells were still largely viable (over 95%); the percentage of damaged cells slightly increased at higher DOPET concentrations. It is interesting to note in this respect that DOPET toxicity is strictly dependent on the cell type. No decrease in cell viability has been observed in cultured intestinal Caco-2 cells up to 900 μ M DOPET [30]; conversely, the molecule at 100 μ M concentration completely inhibits HL60 cell proliferation and leads to more than 50% loss of cell viability either in human prostate cancer cells (unpublished results) or in immortalized renal proximal tubule cells [60].

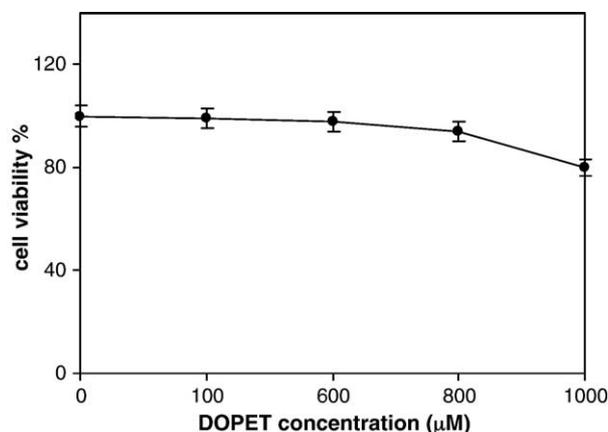


Fig. 1. Effect of DOPET on M14 cell viability evaluated using the trypan blue exclusion test. M14 cells were incubated overnight (18 h) in RPMI medium containing increasing amounts of DOPET. Cell viability was evaluated with the trypan blue exclusion test as described in Materials and methods. Results are expressed as percentage of viability related to control. Data are expressed as mean \pm SE ($n = 3$).

To explore, in deeper detail, dose-and time-response patterns of DOPET treatment, M14 cell viability was evaluated by means of the MTT assay, as described under Materials and methods. As shown in Fig. 2A, M14 cells were able to grow up to 72 h in presence of 100 μ M antioxidant. At the highest employed concentration (1 mM), DOPET exerts a significant toxic effect, with more than 95% of cell death after 72 h treatment. However 24 h incubation with DOPET, up to 600 μ M, did not cause a definitive cell commitment toward a nonproliferating state. Cell growth, indeed, effectively restarted, upon extensive wash and reincubation of M14 cells with DOPET-free medium (Fig. 2B).

It has been reported that olive oil phenolic compounds are absorbed in a dose-dependent fashion in the range employed and actively metabolized in humans [61,62]. Furthermore the characterization of DOPET metabolites, both in rat tissues [20] and Caco-2 cells [27], allows illustration of a metabolic pathway of this molecule, implying the involvement of several cytosolic enzymes such as catechol-*O*-methyltransferase (COMT), alcohol dehydrogenase, aldehyde dehydrogenase, and phenol sulfotransferase [20]. The metabolic fate of this molecule was then evaluated in M14 cells incubated overnight in the presence of [14 C] DOPET. As shown in Fig. 3, an extensive metabolic transformation of DOPET does occur, the major identified metabolite being the methylated derivative, homovanillic alcohol (MOPET), product of COMT activity. Metabolic conversion of DOPET into MOPET was complete after overnight incubation of the cells with DOPET (Fig. 3; open symbols). At the end of the incubation DOPET was no longer detectable in the cells but MOPET was the major species detected. Since in the following experiments irradiation was always carried out after overnight incubation with DOPET, conversion of DOPET into MOPET had been already accomplished at that time.

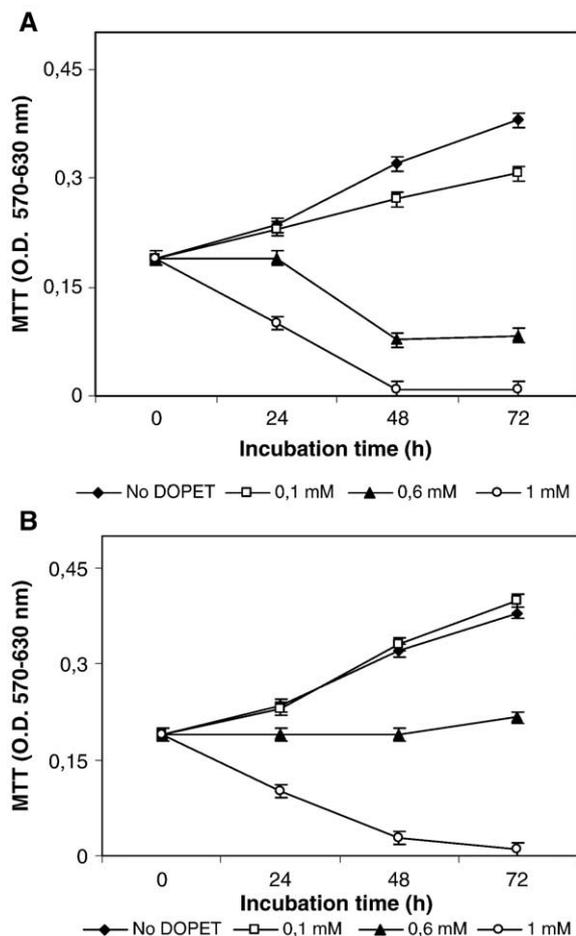


Fig. 2. Effect of DOPET on M14 cells viability evaluated using the MTT assay. (A) M14 cells were incubated as described in the figure in the absence or in the presence of increasing amounts of DOPET. (B) M14 cells were incubated as in A. After 24 h, DOPET was removed, cells were suspended in fresh RPMI medium, and the incubation was prolonged up to 72 h. Cell viability was measured using the MTT assay descriptor in Materials and methods. Values are mean \pm SE ($n = 3$).

The high activity of COMT in UV-irradiated M14 cells, when melanogenesis is stimulated [63], prevents the formation of the other DOPET metabolites [20,64]. It is

has been already reported that MOPET is endowed with a radical scavenging ability comparable to that of its parent compound [64]; therefore, under our experimental conditions, the antioxidant effect of DOPET mainly relies on the formation of its methylated derivative.

UVA radiation gives rise to oxidation products in human melanoma cells, which are counteracted by DOPET/MOPET

Previous results from our [31] and other laboratories [16,48–50] pointed to the role of oxidative stress as a mediator of the biological effects induced by UVA. Therefore, in order to test the ability of DOPET to effectively protect cells against UVA-induced oxidative damage, M14 cells were incubated overnight in the presence of increasing concentrations of this phenol. Cells were then subject to UVA irradiation as described under Materials and methods. At the end of treatment, cells were recovered and several biochemical markers of the oxidative damage were checked.

ROS formation in M14 cells was directly evaluated using the DCFH-DA fluorescence method [52]. Fig. 4 shows that formation of radicals, induced by UVA treatment, was prevented by DOPET with ~40% scavenging activity at 400 μ M concentration. This concentration, not cytotoxic in our cellular system (see Fig. 1), is comparable with the intake of this compound with the Mediterranean diet. For the sake of completion, it should be noted that a decrease in DCF fluorescence intensity could be due to DOPET's ability of either scavenging secondary, reactive radicals or preventing formation of superoxide and/or hydrogen peroxide formed in response to UVA treatment [48–50].

Membrane phospholipids are a major target of oxidative damage and, as already reported [31], in M14 cells UVA induced a dramatic rise in the occurrence of lipid peroxidation products, detected as TBARS, compared to the nonirradiated control. Overnight incubation with DOPET, before UVA irradiation, effectively counteracts

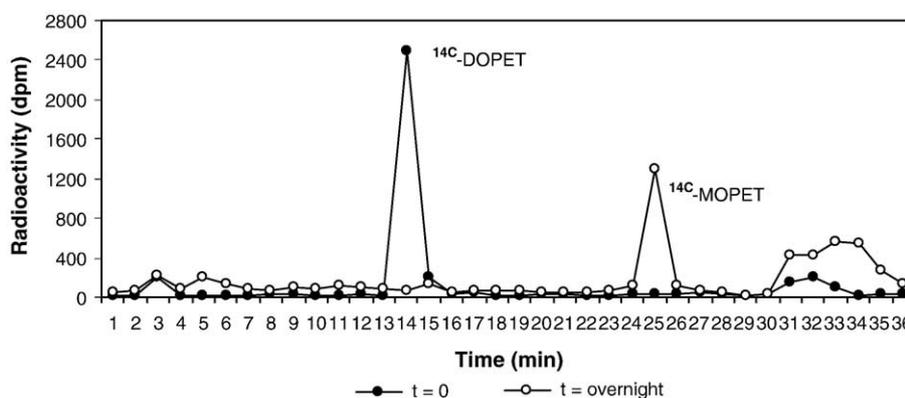


Fig. 3. Metabolic fate of DOPET in M14 cells. HPLC profile of [¹⁴C] molecular species detectable in M14 cells incubated overnight (18 h) in presence of [¹⁴C]DOPET. Samples were treated as reported in Materials and methods. Labeled compounds were identified on the basis of their retention time. Time 0 (full circle); overnight incubation (open circle).

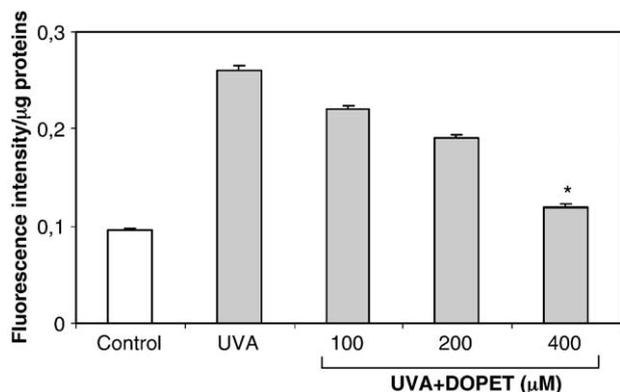


Fig. 4. Effect of DOPET on UVA-induced intracellular ROS production in M14 cells. At the end of an overnight incubation in the presence of increasing amounts of DOPET M14 cells were irradiated with UVA (7.5 J/cm²-dose). ROS production was detected with the fluorescent indicator 2,7-DCFH as indicated in Materials and methods. Data are expressed as means \pm SE ($n = 3$). Asterisk indicates value significantly different from UVA-irradiated sample; $P < 0.05$.

this effect within the 100–400 μ M concentration range (Fig. 5).

It has been reported that riboflavin can be UVA-photoexcited, giving rise to ROS [65]. In this respect we may consider that riboflavin concentration in the medium we used (0.5 nM) was lower, by at least two orders of magnitude, than that present, under physiological conditions, in several tissues and biological fluids [66,67]. Therefore, the effects we observed were never to be considered artifactual, since riboflavin concentration in our biological system was well below its physiological levels commonly observed in vivo. In addition, since UVA irradiation increased TBARS formation even when using PBS as a sample medium (data not shown), we could conclude that in our experimental system UVA irradiation gives rise to ROS through a mechanism which is not crucially mediated by riboflavin, likely because of the very low concentration of this compound in the medium, compared to the in vivo conditions.

DOPET induces apoptosis in human melanoma cells

It is known from the literature that UVA rays are able to induce cell apoptosis in several in vitro systems and the relevant mechanism(s) are critically intertwined with the role of the oxidative stress [68,69]. However UVA treatment of M14 cells does not cause immediate or delayed apoptosis, detectable by DNA fragmentation [31].

As far as DOPET apoptotic activity is concerned, Della Ragione et al. demonstrated that this molecule, at micromolar concentrations, is able to induce apoptosis in proliferating and quiescent HL60 cells, as well as in resting and proliferating peripheral blood lymphocytes [30]. Conversely, two different colon cell lines, Caco-2 and HT29, do not undergo apoptosis upon DOPET treatment [30]. In order to check whether or not DOPET could be involved in the

molecular mechanisms of apoptosis in M14 cells, the activity of caspase 3 was evaluated as a marker. Results, reported in Fig. 6, indicate that UVA treatment itself does not increase caspase 3 basal activity of melanoma cells. This result supports the view that cells normally exposed to sunlight might develop strong mechanisms to protect themselves against damages induced by ultraviolet radiation. As shown in Fig. 6 DOPET exerts a dose-dependent apoptotic effect on melanoma cells. This effect is not related to UVA treatment, since no significant difference in caspase 3 activity was observed between M14 cells, treated with DOPET, with or without UVA irradiation. No effect on caspase 3 activity is detected up to 200 μ M concentration; however, a clear trend pointing toward activation of this protease was observed at higher concentrations of the antioxidant. This result was confirmed by FACS analysis of propidium iodide incorporation and exclusion that shows the appearance of a subgenomic DNA population in M14 cells treated with 1 mM DOPET (data not shown).

Although antioxidants are in general regarded as able to inhibit apoptosis, it has been reported that flavonoids exert apoptosis-inducing activity [70]. A recent paper reports that DOPET induces apoptosis in several cell culture systems, through a p53-independent mechanism [30]. Our present data are highly consistent with the latter report. In order to explain this apparent paradox it is possible to speculate that: (a) the mechanism(s) involving interactions of this *ortho*-diphenol with specific cellular targets might be at the basis of its apoptotic effect; and (b) the above evidence supports the interpretation that the proapoptotic effect of DOPET is not mediated by its antioxidant activity.

DOPET/MOPET protects against protein damage induced by UVA irradiation in melanoma cells

It has been already shown that protein damage is one of the primary and early events on UVA-induced cell injury in M14, preceding both DNA breakage and lipid peroxidation

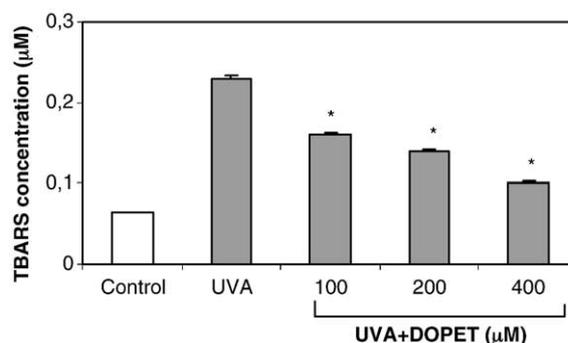


Fig. 5. Effect of DOPET on lipid peroxidation in M14 cells exposed to UVA radiation. Cells were treated as indicated in Fig. 4. TBARS were assayed as reported in Materials and methods. Data are expressed as means \pm SE ($n = 5$). Asterisks indicate values significantly different from UVA-irradiated sample; $P < 0.05$.

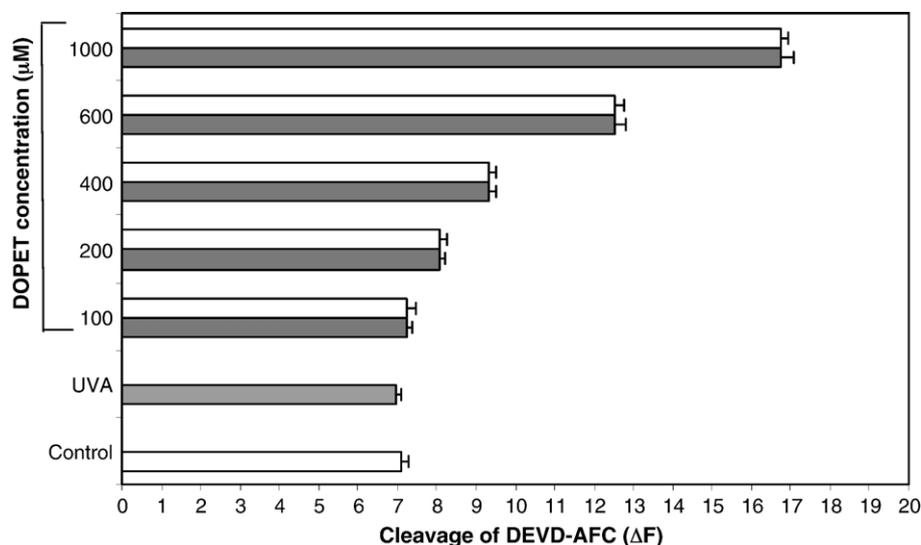


Fig. 6. Measurement of z-DEVD-AFC proteolysis catalyzed by M14 cell extract. M14 cells were treated as reported in Fig. 4. After irradiation, cell lysates were prepared and caspase-3 activity was measured as reported in Materials and methods. Cleavage of z-DEVD-AFC was evaluated fluorometrically (excitation at 375 nm, emission at 530 nm). Data are expressed as means \pm SE ($n = 3$). Irradiated cells, \square ; nonirradiated cells, \blacksquare .

[31]. On the other hand, isoaspartyl formation, to an abnormally high extent, has been observed in human erythrocytes, when these cells are subject, *in vitro*, to an oxidative stress [47]. This condition is particularly evident in the erythrocytes from patients with a deficiency of G6PD, an enzyme critically involved in the maintenance of reducing power in the red blood cells [71]. L-Isoaspartyl residues are specifically and quantitatively recognized and methylated by the enzyme PIMT as the first step of a pathway aimed at the repair of such protein alterations [38,43,72,73]. Because of its peculiar substrate specificity, PIMT is an ideal enzymatic probe for evaluating the extent of L-isoaspartyl formation, both *in vitro* and in intact cells, thus allowing identification and characterization of the mechanism(s) through which cell stresses are able to elicit this kind of protein damage [42,45–47].

In consideration of the antioxidant power of DOPET (and/or DOPET metabolites), we were prompted to study whether this compound was able to prevent the occurrence of isoaspartyl residues, evaluated by PIMT assay, in UVA-irradiated melanoma cells. To this end, M14 cells, after an overnight incubation in presence of increasing concentrations of DOPET, were irradiated with UVA at a 7.5 J/cm² dosage. Cells were then incubated in the presence of [*methyl*-³H]methionine, the *in vivo* precursor of the methyl donor *S*-adenosylmethionine (AdoMet). The incorporation of labeled methyl groups, as protein methyl esters, was used to evaluate the level of L-isoaspartyl residues, as described under Materials and methods. To rule out any spurious effect due to possible interference of DOPET metabolism with PIMT activity, in parallel with protein methyl esterification, AdoMet specific activity was evaluated and the results were expressed as methyl groups incorporated per milligram of cell protein. As shown in Fig.

7A, DOPET treatment effectively prevents the increase in protein methylation, as a marker of protein damage at the aspartyl level, in M14 UVA-irradiated cells.

It is worth noting, in this respect, that the formation of MOPET, through COMT activity, gives rise to *S*-adenosylhomocysteine (AdoHcy) [74], a powerful competitive inhibitor of all AdoMet-dependent methyltransferases. *In vivo*, the extent of such inhibition depends on the AdoMet/AdoHcy ratio (transmethylation potential) and on the relevant K_m and K_i values of the different methyltransferases. Under physiological conditions, AdoHcy inhibitory effect is prevented by its prompt enzymatic hydrolysis into adenosine and homocysteine, a fully reversible reaction catalyzed by AdoHcy hydrolase [75]. However, it has been reported that under pathological conditions, such as hyperhomocysteinemia, intracellular levels of AdoHcy are drastically increased and a significant inhibition of both PCMT-catalyzed protein methylation [55,76] and DNA methylation [77] does occur. Therefore it is possible to hypothesize that DOPET treatment, leading to a metabolic overload of a COMT substrate, increases the intracellular concentration of AdoHcy, thus affecting the PIMT reaction (Fig. 8). As a matter of fact the AdoMet/AdoHcy concentration ratio is decreased in M14 cells treated with 400 μ M DOPET (Table 1) and, although rather slight, this decrease might affect, unpredictably, the protein methylation rate. At a lower concentration of this compound the AdoMet/AdoHcy ratio showed only a slight decrease which however might still affect, unpredictably, the protein methylation rate. To rule out that the decrease in protein methylation, observed in DOPET-treated M14 cells, could be due to PIMT inhibition by AdoHcy, an *in vitro* evaluation of the protein L-isoAsp content was performed. Proteins extracted from UVA-irradiated M14 cells, with or

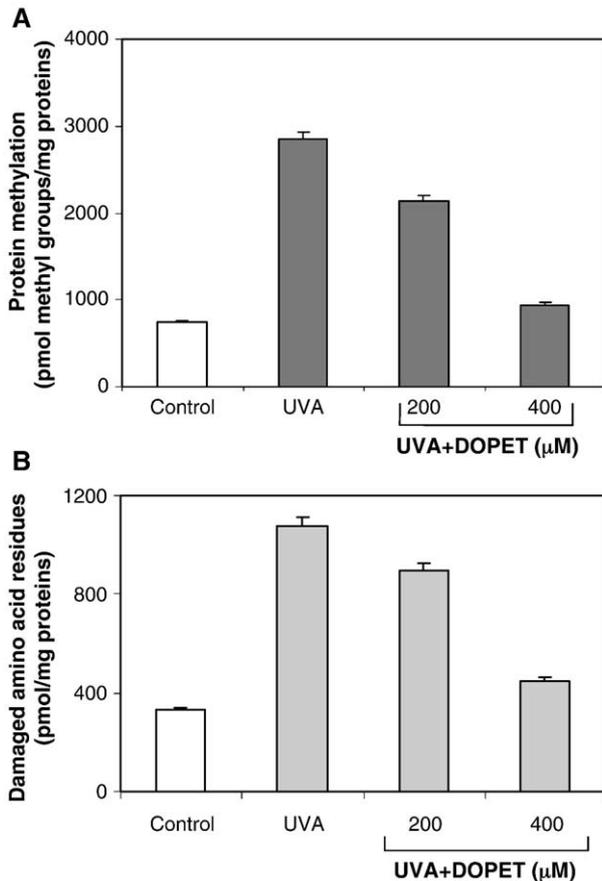


Fig. 7. Effect of DOPET on protein methyl esterification in UVA-irradiated M14 cells. M14 cells were treated as reported in Fig. 4. (A) Analysis in intact cells: cells were incubated in presence of L-[methyl- ^3H]methionine, as described in Materials and methods. Protein methyl esterification was evaluated as the difference between the data of samples incubated with and without protein methylation inhibitors. Protein methylation is expressed as ^3H -methyl groups incorporated/mg protein. The specific activity of intracellular Ado-[methyl- ^3H]Met was evaluated by HPLC analysis of cell extract. (B) In vitro analysis: after treatment, cell lysates were prepared and incubated with recombinant human PIMT and the methyl donor *S*-adenosyl-L-[methyl- ^{14}C]methionine. All results are expressed as the mean \pm SE ($n = 5$); * $P < 0.05$.

without DOPET pretreatment, were methylated in vitro using human recombinant PIMT in the presence of [methyl- ^{14}C]AdoMet as the methyl donor. The methyl-accepting capability of cell proteins in vitro reflects the extent of protein damage induced by UVA in the intact cell [31,42]. Results, in Fig. 7B, confirmed that DOPET effectively prevents formation of L-isoasparthyl residues, in cells irradiated with UVA. Comparison between the in situ (Fig. 7A) and in vitro (Fig. 7B) assays points out that DOPET metabolic conversion into MOPET does not cause an accumulation of AdoHcy up to a limit resulting in a significant impairment of PIMT activity, at least within the DOPET concentration range employed. Consequently, the ability of this enzyme to keep up with the formation of damaged aspartyl residues is well preserved under the experimental conditions employed.

Conclusion

UVA and aspartyl protein damage: A fine balance between oxidation and methylation reactions

Results as a whole showed that, when M14 cells are irradiated with UVA, a significant increase of protein damage, in the form of abnormal L-isoaspartyl residues, does occur, as monitored by the increase in methylatable sites recognized by PIMT. The outcome in terms of the possible consequent modifications of protein function may vary from protein to protein and may crucially depend on both the number and the position of the residues involved [38]. As for the mechanism(s) of this protein damage, we may hypothesize that such an effect of oxidative stress upon Asp/Asn residues is not direct, but must be mediated by rearrangements of the three-dimensional organization of the protein. This view is supported by previous work, which demonstrated that in certain proteins, such as calmodulin, deamidation is accompanied by extensive conformational modifications, resulting in substantial changes of the Stoke's radius [73].

The present results are in line with the notion that oxidative stress plays a role in the generation of damaged/methylatable aspartyl residues in cell proteins. This interpretation is strengthened by the observation that, besides DOPET, other antioxidants, such as vitamin C, effectively smother the tide of both increasing oxidation products and protein damage, in UV-irradiated melanoma cells (data not shown). However, since DOPET does not fully counteract the effects of the irradiation treatment, it is possible to speculate that UVA-induced damage might also be, in part, related to mechanism(s) not directly mediated by oxidative stress.

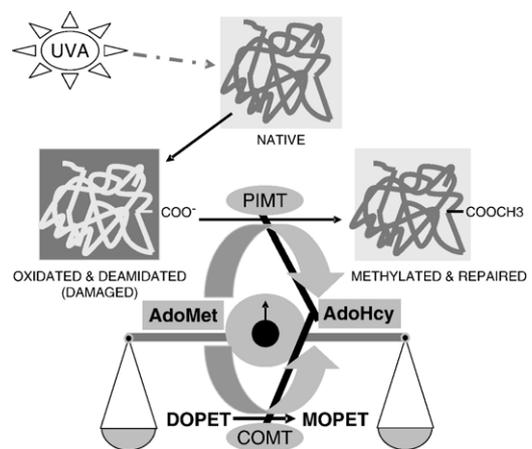


Fig. 8. Overall mechanistic model of action of DOPET in UVA-treated cells. UVA treatment by eliciting oxidative stress is bound to increase protein damage consisting in the formation of L-isoAsp residues, which are recognized and repaired by PIMT. DOPET, in turn, is quantitatively converted into MOPET by COMT, which also consumes AdoMet and produces AdoHcy, just like PIMT. Therefore COMT activity might contribute to the alteration of the AdoMet/AdoHcy ratio (transmethylation potential) and of the balance between oxidative (damaging) events and antioxidant as well as protein repair systems.

Table 1
PIMT specific activity and AdoMet/AdoHcy ratio in M14 cells

Sample	PIMT specific activity (U/mg proteins)	AdoMet/AdoHcy ratio
Control	40.7 ± 5.2	2.8 ± 0.5
200 μM DOPET	37.4 ± 4.1	2.6 ± 0.4
400 μM DOPET	34.3 ± 4.2	2.0 ± 0.5

PIMT specific activity was measured in vitro by a radiochemical enzyme assay in the presence of saturating concentrations of both the methyl donor [*methyl*-¹⁴C]AdoMet and a methyl-accepting protein substrate. 1 U enzyme activity is defined as 1 pmol methyl group incorporated/min. AdoMet and AdoHcy concentrations were determined by HPLC analysis of acid-soluble extracts of M14 cells (see Materials and methods).

In our model system, DOPET is quantitatively converted into methylated derivatives by the AdoMet-dependent COMT. Therefore, our data suggest that compounds derived from DOPET metabolism, more than the molecule itself, are responsible for the antioxidant activity exerted in vivo by olive oil-derived consumer products, including cosmetics and functional foods.

The enzyme COMT, endowed with a rather high specific activity in vivo [74], is involved in the catabolism of several compounds of pharmacological interest, which are largely employed in human therapy [78,79]. In this respect, it has been previously reported that xenobiotics, such as DOPET, undergoing methylation during their metabolism, may interfere with the activity of other AdoMet-dependent methyltransferases, by overproducing AdoHcy, a powerful methyltransferase inhibitor [80]. Although we were able to set up experimental conditions in which DOPET appears to be rather devoid of such a side effect, at least on PIMT, this possibility must be always taken into account when DOPET, or any related substances, is used in a more general setting.

Finally, our results suggest an overall mechanistic model on the balance between damaging, mostly nonenzymatic, reactions and enzymatic protective mechanisms (Fig. 8). According to this model both AdoMet bioavailability for methyltransferases and concentration of AdoHcy, the methyltransferase inhibitor, which is itself a product of methyl transfer reactions, play a role in the regulation of both DOPET metabolism and protein methylation. in vivo. This modulation depends on the [AdoMet]/[AdoHcy] ratio and on the K_m and K_i values, for the methyl donor and the inhibitor, of the two methyltransferases involved [77]. The values of these kinetic constants are slightly more favorable in the case of COMT than PIMT [81]. Therefore, we may expect that, when the [AdoMet]/[AdoHcy] ratio decreases, protein methylation will be relatively hampered, compared to DOPET metabolism, and this effect would increase, the higher the DOPET concentration employed. Therefore, upon UVA irradiation in DOPET-treated cells, a critical interplay between protein oxidation (damage) and methylation (repair) takes place. As for methylation processes, the rates of two different methyl transfer reactions, which control protein repair (PIMT) and DOPET metabolism

(COMT), respectively, can be expected to regulate the balance between protection against oxidative processes and the extent of protein repair.

Acknowledgments

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