Radiosensitizing Effect of Rosmarinic Acid in Metastatic Melanoma B16F10 Cells

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Abstract. Background: Rosmarinic acid is an ester of caffeic acid with interesting biological activities including antioxidant effects and scavenging of oxygen-free radicals. Aim: To determine the potentially paradoxical effect of rosmarinic acid, typically being radioprotective when applied to non-tumorous cells, yet conversely displaying a sensitizing action when applied to metastatic B16F10 melanoma cells. Materials and Methods: The genoprotective effect was studied by means of micronucleus tests for anti-mutagenic activity in which the reduction in the frequency of micronuclei was evaluated using cytokinesis-blocked human lymphocytes. The radioprotective effect was studied via a cell viability test in PNT2 (human prostate epithelium) and B16F10 melanoma cells. Results: Rosmarinic acid exhibits a significant genoprotective capacity (p<0.001) against X-rays with a protection factor of 58%, and a dose reduction factor of 7.2. Cell survival obtained after exposure to 10 Gy of X-rays showed a protection factor of 47.5%, thus eliminating 29.1% of radiation-induced cell death in normal prostate epithelial cells (p<0.001). However, in metastatic B16F10 melanoma cells, rosmarinic acid acted not as a radioprotector, but as a sensitizing agent, increasing cellular death by 42% (p<0.001), with an enhancement ratio of 2.36. Conclusion: Rosmarinic acid has an increased capacity for producing radio-induced damage, and thus a paradoxical damaging effect in melanoma cells. Potentially, research into substances such as rosmarinic acid could help clarify mechanisms that provide protection on healthy normal cells, while exclusively damaging neoplastic cells, thus presenting a new strategy for patients undergoing radiotherapy for cancer.

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It is commonly found in species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae, mainly, Rosmarinus officinalis L. (1). Rosmarinic acid has a number of interesting biological activities, including anti-viral, anti-bacterial, anti-inflammatory and antioxidant effects. The presence of rosmarinic acid in medicinal plants, herbs and spices has beneficial and health-promoting effects. In plants, rosmarinic acid is believed to act as a defence compound. Plant extracts containing rosmarinic acid also have excellent potential as antioxidants for food preservation (1, 2). It is also known to have complement-independent effects such as scavenging of oxygen-free radicals (3, 4). Most recent studies confirm that caffeoyl esters such as rosmarinic acid have a high antioxidant activity, delay vitamin E depletion, reduce pro-inflammatory lysophosphatidylcholine production and prevent the oxidation of low-density lipoprotein (LDL), which is compatible with its anti-inflammatory and anti-atherosclerotic role in pathophysiological conditions (5).

It is known that ionising radiation (IR) generates reactive oxygen species (ROS) in organisms and induce cellular DNA damage, which leads to mutations and chromosomal aberrations (3, 4). Recently, the scavenging ability of certain plant extracts containing several polyphenols, flavones, catechins, and procyanidins against ROS and their inhibitory effects against X- and γ-ray-induced cellular changes were reported in vivo and in vitro (3, 4, 6-8).

In the present study, we examined the radioprotective capacity of rosmarinic acid in non-tumorous cells which disappears and transforms into a sensitizing activity on melanoma cells. Perhaps these opposing effects could offer a new strategy for treating a variety of human cancer types which are resistant to radiotherapy.
Materials and Methods

Chemicals and reagents. Rosmarinic acid (95%) and diosmine were obtained from Extrasyntehse (Genay, France); 82% carnosic acid, eriodictyol, quercetin and green tea extract were supplied by Nutrafur, SA (Murcia, Spain). Amifostine was obtained from Shering-Plough (Ethylol®; Madrid, Spain). Apigenin was obtained from Sigma Co. (Madrid, Spain) and dimethyl sulphoxide (DMSO) was obtained from Merck (Darmstadt, Germany).

Cell lines and culture conditions. The normal epithelial human prostatic cell line (PNT2) was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK), Health Protection Agency Culture Collection (catalog n. 95012613; Salisbury, UK). PNT2 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM) and streptomycin plus penicillin (100 μg/ml and 100 IU/ml, respectively). The metastatic melanoma cell line (B16F10) was kindly provided by Dr. Hearing from the National Cancer Institute (Bethesda, MD, USA). B16F10 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12K (1:1), containing 10% fetal bovine serum (Gibco BRL, Louisville, KY, USA) and 5% penicillin/streptomycin. The cell cultures were kept at 37˚C and 95% relative humidity, in an atmosphere with 5% CO2. Tests were carried out to confirm the absence of Mycoplasma spp. throughout the study. The substances were administered by adding 20 μl to each well of a solution of 10 μM, 20 μM or 40 μM before or after the irradiation with X-rays according to the assay tested.

Genoprotective effects: Micronucleus test (CBMN). Human whole-blood was collected from two healthy young non-smoking female donors into heparinised tubes and mixed well. For before-X-irradiation treatments, 20 ml of these solutions were added to 2 ml of the heparinised human blood to obtain a 20-μM solution, and the samples were homogenized immediately before X-irradiation. For the treatments after X-irradiation, 20 μl of these solutions were added to 2 ml of irradiated human blood (20 μM) which was then homogenised for 10 min after X-irradiation.

Culture technique: After X-irradiation (with addition of substances before and after X-irradiation), the micronucleus assay was carried out on the irradiated lymphocytes with the following cytokinesis-blocking (CBMN) method described by Fenech and Morley (9) and adapted by the International Atomic Energy Agency (10). Briefly, whole-blood samples (0.5 ml) were cultured at 37°C for 72 h in 4.5 ml of F-10 medium containing 15% fetal bovine serum, 1.6 μg/ml phytohaemagglutinin, 1% penicillin/streptomycin and 1 μg/ml of glutamine. Forty-four hours after initiation of the lymphocyte cultures, 150 μl of colchicine B were added at a concentration of 6 μg/ml. At 72 h, the lymphocytes were treated with hypotonic solution (KCl, 0.075 M) for 3 min and fixed using methanol/acetic acid (3:1). Air-dried slide preparations were made and stained with May-Grünwald Giemsa 24 hours later. Each experiment was repeated on three occasions.

Scoring of micronuclei: The numbers of micronuclei in at least 3,000 cytokinesis-blocked cells for each case were determined by two specialists who analysed the slides of all groups in a double blind. These slides were examined using a Ziss light microscope (Oberkochem, Germany) with ×400 magnification for surveying the slides and ×1,000 magnification to confirm the presence of MN in the cells.

Radioprotective effects: (MTT) test. To analyze for the radioprotective effects of substances on PNT2 and B16F10 cell viability and survival, we used the MTT assay. Briefly, the cultures were incubated in 200 μl growth medium and allowed to adhere for 24 h. After treatment, supplemented growth medium and 50 μl of MTT (5 mg/ml) were added to each well and microplates were further incubated in a 5% CO2 atmosphere at 37°C for 4 hours. After centrifugation to carefully remove the medium and non-metabolized MTT, 100 μl of DMSO were added to each well to solubilize the MTT formazan produced by the cultured cells. After shaking for 30 min at room temperature, the plates were read with a Multiskan MCC/340P spectrophotometer (MTX Lab Systems, Virginia, USA) using 570 nm for the test reading and 690 nm as the reference wavelength. The negative control well containing medium was used for the baseline zero. Each experiment was repeated thrice.

Treatment and irradiation. The samples were exposed to X-rays using an Andrex SMART 200E instrument (Yxlon International, Hamburg, Germany) operating at 200 kV, 4.5 mA, focus-object distance 36 cm at room temperature. The radiation doses were monitored by a UNIDOS® Universal Dosimeter with PTW Farm® ionization chambers TW30010 (PTW-Freiburg, Freiburg, Germany) in the radiation cabin and the dose of radiation of X-rays was confirmed by means of thermoluminescent dosimeters (TLDs) (GR-200®; Conqueror Electronics Technology Co Ltd, P.R. China). The Ministry of Industry and Energy (Spain) supplied the TLDs and also measured their absorbed doses after the experiments. In the CBMN study of human lymphocyte cells, 2 Gy of X-rays were administered, whereas different doses of X-rays (4, 6, 8, 10 and 0 Gy as control) were used in the MTT cell viability assay.

Statistical analysis. In the genoprotective study, the degree of dependence and correlation between variables were assessed using analysis of variance complemented by a contrast of means (p<0.05). Quantitative means were compared by regression and linear correlation analysis. In addition, we used the formula described by Sarma and Kesavan (11) to evaluate the factor of protection: factor of protection (%)=\[(F_{control irradiated} – F_{treated and irradiated})/F_{control irradiated}\]×100.

Where Fcontrol irradiated is the frequency of micronuclei in untreated but irradiated blood lymphocytes, and Ftreated irradiated is the frequency of micronuclei in blood lymphocytes treated with substances and irradiated (administered before and after X-irradiation).

The dose-reduction factor (DRF) was calculated as the ratio of radiation dose required to produce the same biological effect in the presence and absence of the radioprotector. We used the formula described by Hall (12): DRF=dose of reduction in the presence of radioprotector/doses of radiation in the absence of the radioprotector, to produce a given frequency of micronuclei.

In the radioprotective study, an analysis of variance (ANOVA) of repeated means was carried-out to compare the percentages of surviving cells in the cultures with different concentrations of the various compounds, complemented by least significant difference analyses to contrast pairs and means. The analyses were carried-out by logarithmically transforming the data to comply with ANOVA conditions.

To determine the protection factor (PF), the formula for the factor protection (11) modified for the analysis of growth inhibition at 10 Gy and incubated 48 h was used: PF (%)=[(MT–MC)/MC]×100, where MT is the mortality of the cells irradiated and treated with...
the substances studied; and MC is the mortality of the irradiated control cells.

The efficacy of a sensitizer is conveniently expressed in terms of the enhancement ratio (ER). This is defined as the ratio of doses in the absence and in the presence of the drug which produce the same biological effect (12). We used the ER of rosmarinic acid for the cells irradiated with 10 Gy and incubated for 48 hours.

**Results**

In the genoprotective study performed, administration of the different substances studied showed no significant differences in the frequency of micronuclei produced compared to the non-irradiated controls, indicating an absence of genotoxic effects of these substances at the tested concentrations. Figure 1 shows the influence of treatments before X-ray irradiation on the frequency of micronuclei in non-irradiated and irradiated human lymphocytes, which permits a comparison of the potential genotoxicity of X-rays (control irradiated) versus its anti-mutagenic capacity in the presence of the different substances assayed. In non-irradiated human lymphocytes, all compounds expressed the same level of micronuclei frequency as the control, indicating the absence of genotoxicity. In irradiated human lymphocytes, the order of treatments administered before X-irradiation, from lowest to highest level of micronuclei was as follows: rosmarinic acid < carnosic acid = apigenin < diosmine < dimethyl sulphoxide < irradiated control < eriodictyol = quercetin ($p<0.001$).

Figure 1 also shows the influence of different substances administered after X-irradiation on the frequencies of micronuclei. The frequencies are higher than in treatments before X-ray irradiation. It is clear that carnosic acid has significant anti-mutagenic activity. Rosmarinic acid demonstrates a low degree of radioprotection, while the sulphur-containing compounds DMSO and amifostine lacked X-ray radioprotective capacity. The order of genoprotection from lowest to highest level against micronuclei induced by irradiation was: carnosic acid < apigenina < diosmine < rosmarinic acid = green tea extract < dimethyl sulphoxide = amifostine = irradiated control < eriodictyol = quercetin ($p<0.001$).

Figure 2 shows the values of these protection capacities, the orders of efficacies being carnosic acid = rosmarinic acid > apigenina < diosmine > amifostine > green tea extract > dimethyl sulphoxide for treatments before X-ray irradiation and carnosic acid > apigenina > rosmarinic acid = diosmine > green tea extract > dimethyl sulphoxide > amifostine for treatments after X-irradiation. Figure 2 also shows the DRFs of the tested substances, reflecting the differences in effects between carnosic acid and rosmarinic acid in relation to the time of substance administration (i.e. before or after exposure to radiation).

In the radioprotective study, administration of the different substances studied led to no significant differences in cell growth compared to the non-irradiated controls, indicating the absence of cytotoxic effects of these substances at the concentrations tested. Radiation-alone caused a dose-dependent and time-dependent decrease in cell viability of both PNT2 and B16F10 melanoma cells ($p<0.001$). Figure 3 shows the cell survival of PNT2 cells as assessed by the MTT cell viability test. For non-irradiated cells, all substances led to the same level of cell survival as the untreated controls, demonstrating an absence of toxicity. For the irradiated PNT2 cells, all substances led to an increase in cell survival at the highest dose (10 Gy) used, which is an expression of radioprotective capability at the two concentrations of the test substances used for the two study periods ($p<0.001$), with carnosic acid and rosmarinic acid having the highest PF of 56% and 47.5%, respectively.
Figure 4 shows the cell survival of B16F10 melanoma cells indicating significant differences when compared with results obtained with non-tumourous PNT2 cells. At 10 Gy, inhibition of growth is higher and an absence of radioprotection of rosmarinic acid and carnosic acid for the cells is revealed. This increased inhibition in cell growth could be interpreted as a sensitizing effect at high concentrations of rosmarinic acid \((p<0.001)\), reaching 42% and an ER of 2.36.

Figure 5 shows the cell survival of B16F10 melanoma cells following administration of a mixture of the two most effective radioprotective substances (rosmarinic acid plus apigenin and carnosic acid plus apigenin) at two different concentrations \((10 \, \mu M + 10 \, \mu M \text{ and } 20 \, \mu M + 20 \, \mu M, \text{ respectively})\). These led to similar results and confirm significant differences from those obtained from B16F10 melanoma cells. However, under these conditions, we determined a significant radioprotective capacity \((p<0.001)\), with a cell survival higher than 95% in irradiated PNT2 cells.

**Discussion**

Ionizing radiation causes a high level of generation of hydroxyl radicals \(\textit{in vivo}\), by homolytic cleavage of body water or of endogenous hydrogen peroxide (formed by reduction of the superoxide anion) by two mechanisms: the Haber-Weiss and Fenton models. The hydroxyl radical is the most cytotoxic of all these so far described, with an estimated half-life of 10-9 s, the high reactivity of this radical implies immediate reaction where it is generated (13, 14). Thus, when hydroxyl radical generation is massive, as with X-irradiation, the cytotoxic effect increases through the interaction of these radicals with cell phospholipid structures, inducing peroxidation processes and the generation of lipoperoxy radicals, which may be regarded as a delayed reaction by ionising radiation (5, 13).

Currently, the ability of different substances to prevent genotoxic damage and their anti-mutagenic capacity is measured in terms of the reduction of these ROS (2-4). We have used the CBMN test to evaluate the genoprotective capacity of several compounds. In addition, we described how some pure flavonoids (diosmin and apigenin) and polyphenolic extracts have a greater capacity than traditional radioprotectors, for example sulphhydryl compounds (dimethyl sulphoxide and amifostine), against both X-rays \(\textit{in vivo}\) a (6, 8, 15) and \(\gamma\)-irradiation \(\textit{in vitro}\) (3, 7). We described how these protective capacities depend on both the degree of polymerization and solubility of the substances assayed, since these modify their bioavailability (4, 6, 7). Reflecting the findings of other authors (18), we have observed that the antioxidant substances contained in different polyphenolic extracts of olive leaf (\(\textit{Olea europaea}\)) (8, 15) and citrus fruits (citroflavonoids) (6-8) have greater protective power when administered alone.

Our results suggest that the anti-mutagenic effect observed is proportional to the antioxidant capacity, although it is also dependent on bioavailability characteristics in the medium assayed. Accordingly, we observed that the flavan-3-ols had the greatest protective capacity of all the polyphenols (6, 7), while other flavonoids with a greater anti-neoplastic and anti-proliferative capacity had a lower antimutagenic capacity (16-19). Continuing the search for compounds with greater antioxidant capacity, we have described other substances that show greater genoprotective capacity with a different chemical structure, rosmarinic acid and carnosic acid (2, 5). However, the present study shows that the substances assayed in the post-X-irradiation treatment do not follow the criteria mentioned above. Indeed their protective capacity is less than when used in the pre-X-irradiation treatment, underscoring the importance underlying the time of administration.

When the phenolics were added after X-irradiation, the only ROS present in the cells, according to the half-life of
superoxide anion and hydroxyl radicals, were lipoperoxy (R-OO.) and other radicals (R., RO., etc.), which are responsible for continuous oxidative chromosomal damage. In addition, ionising radiation is known to enhance lysosomal enzyme secretion and arachidonic acid release from membranes through lipoxygenase, cyclooxygenase and phospholipase activities, leading to an increase in inflammatory cell response. Lipoxygenase and cyclooxygenase are also involved in other processes (endoperoxide formation, prostaglandins, leukotrienes, etc.). Under these complex oxidative stress conditions, it is very difficult to make a structural linear evaluation of the experimental data obtained from the anti-mutagenic activity measured in different post-irradiation treatments, however, some considerations are possible (2, 5). The results obtained in the post-irradiation treatments show that there was a significant decrease in the radioprotective-anti-mutagenic activity of rosmarinic acid, a water-soluble compound, with results comparable to those for amifostine and dimethyl sulphoxide which lack X-ray radioprotective capacity. However, carnosic acid a liposoluble compound maintained its genoprotective capacity.

Our results show that this pathway of lipoperoxy radical elimination is quantitatively greater when carnosic acid is administered immediately after exposure to ionising radiation. According to our previous findings, the present study indicates that the only compound with a significant radioprotective effect (anti-mutagenic capacity) in administration after X-irradiation is the liposoluble compound (carnosic acid) (3, 4).

The results obtained concerning radioprotective effects (anti-mutagenic activity) for rosmarinic acid were close to those reported by other authors on its antioxidant activity (2, 5, 19, 20), obviously, the degree of efficacy of rosmarinic acid and carnosic acid depends on its structure. It is known that the capacity to scavenge hydroxyl radicals is principally based on the combination of conjugated structures in the polyphenolic skeleton, mainly the o-dihydroxy-phenol or catechol structure; thus the presence in rosmarinic acid and carnosic acid of two catechol groups conjugated with a...
carboxylic acid group increases its antioxidant activity in aqueous media. In fact, according to these structural considerations, the anti-mutagenic activity of rosmarinic acid and carnosic acid given before X-irradiation is consistent with their antioxidant properties and specific activities as a free radical scavenger. These data also confirm the higher anti-mutagenic activity of rosmarinic acid over those of amifostine and dimethyl sulphoxide (4).

Similarly, our results show a significant radioprotective effect of rosmarinic acid and carnosic acid on human prostate epithelial cells. It has been reported that these substances have minimal effects on non-tumorigenic prostate epithelial cells treated with increasing concentrations (21). We thus expected a protective effect on cell survival against damage induced by ionising radiation, similar to that obtained in the study of genoprotection.

Surprisingly, we determined a lack of radioprotective capacity on metastatic melanoma B16F10 tumour cells, and even showed a radiosensitizing effect induced by both substances (rosmarinic acid and carnosic acid). The anti-tumour capacity of rosmarinic acid and carnosic acid was established in different human tumours and human cell lines (21-23), in human melanoma (24), and also in the B16F10 cell line (21, 25-27). Carnosol has been reported to inhibit the invasion of highly metastatic mouse melanoma B16F10 cells in vitro, leading to the conclusion that carnosol targets matrix metalloproteinase-mediated cellular events in cancer cells and provides a new mechanism for its anticancer activity (26, 28). Additionally, Visanji et al. postulated that the induction of G2/M phase cell-cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and B1 levels (29).

From our experience, in the study of growth inhibition of different types of melanoma cells induced by polyphenolic substances (16-19), we had not expected a complete loss of radioprotective capacity of the tested substances. Also unclear is the mechanism by which rosmarinic acid loses its radioprotective capacity in tumor cells. This could offer a new application to selectively-protect healthy cells from ionising radiation without protecting tumour cells when both...
types of cells are jointly exposed in the same field. In this case, normal cells may benefit from the radioprotective effects of these compounds; whereas in contrast, the tumor cells suffer a radiosensitizing effect. Therefore, according to Yesil-Celiktas et al., rosmarinic acid alone or in combination with other anticancer drugs (or ionising radiation) may offer a good strategy for the treatment of a variety of human cancer types that are resistant to chemotherapy or radiotherapy (24).

Although the mechanism by which rosmarinic acid loses its radioprotective capacity in mouse melanoma B16F10 cells is obscure, one possible mechanism could be related to melanogenesis in these cells. The effect of some botanical extracts on melanogenesis has been documented (30) and studies have shown that some flavonoids such as naringin (31), caffeoyl compounds, such as caffeic acid (32) and its dimer rosmarinic acid (5, 32), stimulate melanogenesis in mouse B16F10 melanoma cells. We have previously described the increase in tyrosinase activity and expression in mouse melanoma B16F10 cells after 48 h stimulation with rosmarinic acid and compared these with corresponding levels in negative controls. In fact, upon visual inspection of cell pellets from samples of B16F10 melanoma cells treated with rosmarinic acid, a darker colour was noticed compared to the control samples which may be explained by increased melanin content in the treated cells (5). In accordance with our description, rosmarinic acid has been postulated to induce melanogenesis (increased melanin content and tyrosinase expression) in a concentration-dependent manner, probably through protein kinase A activation signalling (33).

It is believed that the glutathione (GSH) redox system may be essential for the production and for the attenuation of abnormal melanin production, with GSH thought to be involved in the regulation of melanin synthesis during melanogenesis. Furthermore, GSH and GSH-related enzymes including gamma-glutamylcysteine lyase and glutathione-S-transferase are important antioxidant defences responsible for maintaining cellular redox balance with capacity to eliminate the ROS induced by ionising radiation (32, 33).
It has been reported that the administration of caffeic acid ester, to participate in melanogenesis, results in intracellular GSH depletion, increased ROS in B16F10 melanoma cells, induction of apoptosis in B10F16 cells and in vivo B16F0 tumor growth inhibition. There is a concomitant decrease in alanine aminotransferase activity and an increase in the level of malondialdehyde, reflecting a decrease in capacity for lipid peroxidation and also a decrease in the free thiol content in liver and kidney (32, 33). Similarly, it is well-known that the highly metastatic B16F10 cell line has a lower superoxide dismutase activity (superoxide radical scavenging) than other less metastatic lines (34, 35). All these mechanisms are modified during melanogenesis and they are also responsible for the endogenous radioprotective capacity of cells exposed to ionising radiation.

Therefore, in our study in normal epithelial cells (PNT2), the rosmarinic acid and carnosic acid act as antioxidants which can eliminate the excess free radicals induced by ionising radiation, in conjunction with the intracellular redox defensive system. However, in B10F16 melanoma cells, melanogenesis is activated, leading to a change in the equilibrium of glutathione and cysteine lyase to facilitate melanin production, which could compromise the intracellular redox defence system. Since this involves a reduction in the scavenging levels of the superoxide radicals together with a loss of part of the antioxidant capacity of rosmarinic acid/carnosic acid, it causes an elevation of intermediate elements, such as intracellular hydrogen peroxide, which produces greater cell damage. Although this mechanism of radiation sensitization is not clear and more detailed studies are needed, this effect could be presented as enhancing the damage induced by ionisation and therefore, portray a paradoxical protective effect of the antioxidant substances towards the melanoma cells. Studies on substances like rosmarinic acid could help clarify mechanisms allowing protection of healthy normal cells while exclusively injuring neoplastic cells, thus presenting a new strategy for patients undergoing radiotherapy for cancer.

Conflicts of Interest
None.

Acknowledgements
This study was supported by a grant from the National Spanish R&D Programme CENIT of the Spanish Ministry of Science and Technology named “Industrial Research Diets and Food with Specific Features for the Elderly” (Acronym: SENIFOOD); D.G. Achel was able to take part in this study because of a sponsored fellowship (GHA/0021) from the International Atomic Energy Agency (IAEA) and A. Olivares thanks to a grant from the Seneca Foundation (Coordination Research Centre of the Region of Murcia, Spain).

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