

Response of cell cycle/stress-related protein expression and DNA damage upon treatment of CaCo2 cells with anthocyanins

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Anthocyanins are a class of flavonoids, widely spread throughout the plant kingdom, exhibiting important antioxidant and anti-inflammatory actions as well as chemotherapeutic effects; nonetheless, little is known about the molecular mechanisms by which these activities are exerted. The present study is aimed at investigating molecular mechanisms involved in the chemotherapeutic effects induced by both cyanidin-3-*O*- β glucopyranoside (CY3G) and its aglycon form, cyanidin chloride (CY), in human colon cancer cells (CaCo2). The effect on cell growth, reactive oxygen species (ROS) formation and cell cycle/stress proteins modification, including ataxia teleangiectasia mutated protein (ATM), p53, p21, 8-oxoguanine DNA glycosylase (OGG1), 70 kDa heat shock protein (HSP70) and topoisomerase II β , as well as on DNA fragmentation, was determined. CY and CY3G treatment affect cell growth and cell proliferation, this latter in a moderately dose-dependent way. Interestingly, ROS level is decreased by any concentration of CY and, only at the lowest concentration, by CY3G. Moreover, the two molecules exert their activities increasing ATM, topoisomerase II, HSP70 and p53 expression. The analysis of DNA fragmentation by Comet assay evidences: (1) a dose-dependent increase in DNA damage only after treatment with CY3G; (2) a more evident trend in the DNA fragmentation when the treatment is performed on agarose embedded cells (cellular atypical Comet); (3) a highly dose-dependent DNA fragmentation induced by CY when the treatment is carried out on agarose embedded naked DNA (acellular atypical Comet). The present findings substantiate a possible chemotherapeutic role of anthocyanins and suggest that CY and CY3G act on CaCo2 by different mechanisms, respectively, ROS-dependent and ROS-independent.

Anthocyanins: CaCo2: Cyanidin: Cell cycle/stress-related proteins: Comet assay

Within the last decade the interest of the researchers on plant natural products has exponentially grown due to their multifactorial capability in biasing human health. Among these substances, anthocyanins, pigments responsible for the brilliant colours of some fruit and vegetables, have been widely investigated. The colour of anthocyanins differs from pink to purple depending on both the differences in their chemical structures and the presence of natural copigments. Interestingly, DNA and RNA also have this effect for an intercalating phenomenon between two adjacent base pairs that provides proper sites for π - π hydrophobic stacking⁽¹⁾. Anthocyanins belong to the widespread class of phenolic compounds collectively named flavonoids and are the most important group of water-soluble plant pigments, closely related to several potent DNA intercalators^(2,3). These aromatic compounds derive from the flavylium cation with variation according

to the hydroxylation and/or methoxylation patterns of the α - and β -rings, as well as to the possible glycosylation of the hydroxyl group at the 3 and/or 5 position. Anthocyanins have been found to be absorbed unmodified from the diet and to be incorporated by cultured cells, both in the plasma membrane and in the cytosol⁽⁴⁾.

Extensive *in vitro* and *in vivo* studies have demonstrated the strong free radical-scavenging properties of anthocyanins, their lowering effect on the risk of CHD and their capability to prevent CVD and neurodegenerative diseases^(5–8), mutagenesis and carcinogenesis^(8–10). Some authors suggest that these functions may be mostly exerted by cyanidin-3-*O*- β glucopyranoside (CY3G), the main anthocyanin present in many fruits such as pigmented orange, berry fruits and grapes^(11,12). Other authors have demonstrated the importance of the *ortho*-dihydroxyphenyl structure of aglycon in exerting

Abbreviations: ATM, ataxia teleangiectasia mutated protein; CY, cyanidin chloride; CY3G, cyanidin-3-*O*- β glucopyranoside; DMSO, dimethyl sulphoxide; HSP70, 70 kDa heat shock protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OGG1, 8-oxoguanine DNA glycosylase; ROS, reactive oxygen species; TDNA, percentage of the fragmented DNA; TMOM, tail moment.

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the enounced activities⁽⁹⁾. Anthocyanins have also been widely reported to inhibit the growth of some human, mice and rat cancer cells^(2,13–16), and to induce apoptosis in different human cancer cell lines^(10,17–19) and in Jurkat T cells in which p53 and bax protein expression is modulated. In addition, other authors have indicated a linkage between the biological effects induced by anthocyanins and their capability to form adducts with DNA⁽²⁰⁾. Structure activity studies have indicated the presence of vicinal hydroxyl substituents at phenyl β -ring as a crucial requirement for target interaction^(9,21), suggesting that the interference of the anthocyanins with different cascades involved in the regulation of cell growth and the induction of apoptosis in cancer cell lines could depend on the substitution pattern at the β -ring.

However, which form of the anthocyanins (i.e. CY3G or cyanidin chloride (CY)) exerts the higher antioxidant, pro-oxidant or chemotherapeutic activity and the molecular mechanisms related to these effects need to be still elucidated.

In the present study some of the possible molecular mechanisms involved in the chemotherapeutic effects exerted by CY3G and its aglycon, CY, upon treatment of human colon cancer cell line (CaCo2) were examined. CY3G and CY were chosen in order to test the involvement of the substituents at the phenyl β -ring of these molecules.

With this aim we investigated the effect elicited by anthocyanins on proliferation, DNA fragmentation and reactive oxygen species (ROS) formation in CaCo2 cells. Contextually, the expression of some cell cycle DNA repair-related proteins (ataxia telangiectasia mutated protein (ATM), p53, p21, topoisomerase II β , 8-oxoguanine DNA glycosylase (OGG1) and 70 kDa heat shock protein (HSP70)) was investigated. OGG1 is the only protein able to remove from DNA 7,8-dihydro-8-oxoguanine, an abundant mutagenic lesion caused by the exposure to free radicals and ROS^(22,23). The level of HSP70, an anti-apoptotic stress-induced chaperone protein, was investigated considering the pivotal role elicited by its enhanced expression in tumorigenesis as well as in cell proliferation and differentiation^(24,25). The present study appears to be helpful for the improvement of knowledge related to the possible use of CY and CY3G in chemotherapy.

Materials and methods

Chemicals

CY and CY3G (purity 99 %) were purchased from Polyphenols Laboratory. All other chemicals were purchased from Sigma.

Cell culture and treatments

Human colon carcinoma cells (CaCo2), obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's modified essential medium (Gibco BRL, Life Technologies) supplemented with 10 % foetal calf serum, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, streptomycin (50 μ g/ml) and penicillin (50 U/ml). The treatments were performed at sub-confluence and the cells were maintained for 24 h with different concentrations (25, 50, 100, 200 μ mol/l) of freshly prepared CY and/or CY3G. The concentration range used in the present study was shifted towards higher levels (raising it up to 200 μ mol/l)

after testing lower concentrations (5 and 10 μ mol/l), which did not exhibit any damaging effect in our experimental model (data not shown). The aglycon was dissolved in sterile PBS plus 0.1 % dimethyl sulphoxide (DMSO), while CY3G was solubilized in sterile PBS. Both solutions were kept in the dark as stock solutions and were diluted to the final concentrations in the culture medium at the time of the treatment. At the end of the treatment the cells were scraped, washed with PBS and immediately utilized for the analysis.

Both untreated and 0.1 % DMSO alone-treated cells were cultured to be considered as control cells. Since in all the tests performed in the present investigation the values obtained with untreated and 0.1 % DMSO treated CaCo2 cells were similar, they were counted all together as means and standard deviations.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide bioassay

In order to monitor cell viability, CaCo2 cells were seeded at 1×10^4 cells per well in a ninety-six-well, flat-bottomed microplate, as previously reported^(22,26). Cells were incubated at 37°C in a humidified 5 % CO₂–95 % air mixture and treated with CY and CY3G as reported earlier. Cell growth medium containing the tested molecules was removed and 180 μ l fresh medium was added before incubating at 37°C for 4 h with 20 μ l 0.5 % 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS. Afterwards the supernatant was removed and replaced with 100 μ l DMSO and 20 μ l 3 % (w/v) dodecyl sulphate in water. The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, DAS, Italy) at $\lambda = 550$ nm. The results have been reported as percentage of cell viability with respect to control cells, whose absorbance was considered to be due to 100 % viable cells.

Clonogenic test

Clonogenic survival test was accomplished by plating 100 cells/dish. Cells were treated for 24 h with the same concentration of CY and CY3G as stated earlier. At the end of the treatment, cells were washed twice with PBS; then fresh medium was added and after 7 d the formed cell colonies were stained (crystal violet) and counted. The colony-forming efficiency was calculated as a percentage with respect to control cells.

Reactive oxygen species measurement

An assay was used to evaluate the ability of CaCo2 cells to modulate the level of ROS after 24 h exposure to CY and CY3G. The assay was performed, as previously described⁽²⁷⁾, by using the fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate. 2',7'-Dichlorodihydrofluorescein diacetate diffuses through the cell membrane; in the cytosol it is enzymatically hydrolysed by intracellular esterases and oxidized to the fluorescent 2',7'-dichlorofluorescein in the presence of ROS. The fluorescence (corresponding to the radical species-oxidized 2',7'-dichlorofluorescein) was monitored spectrofluorometrically using a Hitachi F-2000 spectrofluorimeter (Hitachi): $\lambda_{\text{excitation}} = 488$ nm, $\lambda_{\text{emission}} = 525$ nm. A positive control was performed in each experiment by treating

the cells with 200 μM - H_2O_2 for 20 min before adding 2',7'-dichlorodihydrofluorescein diacetate to the cells.

The total protein content was evaluated for each sample according to Bradford⁽²⁸⁾. The results are reported as fluorescence intensity per mg protein and compared to the relative controls.

Comet assay

Typical assay. The presence of DNA fragmentation single-cell gel electrophoresis (Comet assay) was performed, according to a modification of the protocol taken from Singh *et al.*⁽²⁹⁾, on CaCo2 cells treated for 24 h as above earlier. At the end of the procedures, the samples were scored using a Leica fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. Dedicated software (Leica-QWIN) allowed the analysis and the quantification of DNA damage by measuring the level of DNA damage as: (1) percentage of the fragmented DNA (TDNA) and (2) tail moment (TMOM) representing the product of the distance between head and tail (TD) and TDNA.

The results are presented as TMOM, since it is considered to be the most comprehensive and meaningful Comet parameter.

Atypical assay. Cells (1×10^5), suspended in 10 μl PBS, were mixed with 65 μl 0.7% low melting agarose, loaded on to 1% normal melting agarose-preloaded microscope slides and allowed to solidify at 4°C for 5 min. The slides were then covered with a third layer of 75 μl low melting agarose and divided into two groups: the first group was used for the cellular version and the second for the acellular version of the atypical Comet assay.

Cellular atypical Comet assay

The sandwich gels, prepared on the microscope glass slides, were located in small chambers containing the two flavonoids (CY and CY3G) in PBS taken singularly in the concentration range 25–200 $\mu\text{mol/l}$. The samples were kept for 30 min, in the dark, in a water bath at 37°C. Then the slides were washed with PBS and immersed in lysis solution (1% *N*-lauril-sarcosine, 2.5 mol/l NaCl, 100 mmol/l Na_2EDTA , 1% Triton X-100, 10% DMSO, pH 10) at 4°C for 1 h. Afterwards the samples were equilibrated in electrophoresis buffer to allow DNA unwinding, then they were electrophoresed, neutralized, stained and visualized with a Leica fluorescence microscope. The DNA images were acquired to quantify the damage according to the parameters reported earlier for the typical Comet assay.

Acellular atypical Comet assay

The sandwich gels were placed in lysis solution (1% *N*-lauril-sarcosine, 2.5 M-NaCl, 100 mmol/l Na_2EDTA , 1% Triton X-100, 10% DMSO, pH 10) for 1 h at 4°C. After two washings with PBS, each slide containing naked DNA embedded in the agarose was located in a small chamber. At this stage, the treatments with 25–200 $\mu\text{mol/l}$ CY or CY3G in PBS started. The samples were kept for 30 min, in the dark, in a water bath at 37°C. Afterwards the slides were electrophoresed and treated as already reported for the cellular version.

Hydrogen peroxide treatment

For the two versions of the atypical Comet assay some samples were treated with H_2O_2 (200 $\mu\text{mol/l}$) either together with 50 $\mu\text{mol/l}$ CY/CY3G (30 min, 37°C), or after treatment with the two flavonoids (30 min CY/CY3G, washing, 30 min H_2O_2 at 37°C).

Immunoblotting analysis

CaCo2 cells exposed for 24 h to the different treatments were washed with PBS, scraped, centrifuged and suspended in lysis buffer (1 mmol/l EDTA, 10 mmol/l Tris, pH 7.4, 0.5 mmol/l phenylmethylsulphonyl fluoride). The debris was removed by spinning at 10000 *g* at 4°C for 20 min. For each sample an equal amount of denatured proteins (50 μg per lane), measured according to Bradford⁽²⁸⁾, was loaded into 8 or 12% SDS–polyacrylamide (SDS–PAGE) minigels and electrophoresed at constant voltage (100 V), according to Laemli⁽³⁰⁾. The blotting on nylon membranes was performed overnight with a constant current of 30 mA and processed as previously described⁽³¹⁾. All primary monoclonal and secondary peroxidase-conjugated antibodies were purchased from Santa Cruz (CA, USA) except for OGG1 that was purchased from Assay Designed Inc. (USA); all the concentrations employed were set up experimentally. The antigen–antibody complexes were evidenced by the ECL-Plus system (Amersham Biosciences) followed by contact with hyperfilm™ ECL radiography (Amersham Biosciences). Autoradiographies were scanned and analysed using a Kodak image system and the level of proteins was quantified by arbitrary densitometric units. The values reported have been normalized with respect to β -actin levels (internal control).

Statistical analysis

Each experiment was repeated at least three times in triplicate and the mean and standard deviation for each value was calculated (three independent measures). Statistical analysis of results was performed using one-way ANOVA test plus Dunnett's test by the statistical software package SYSTAT, version 9 (Systat Inc., Evanston, IL, USA). Differences were considered significant at $P \leq 0.05$.

Results

Colon carcinoma cells exposure to cyanidin chloride and cyanidin-3-O- β glucopyranoside inhibits cell growth and production of reactive oxygen species

The results of the clonogenic test and MTT assay (Fig. 1(A), (B)) indicate that CY and CY3G reduce cell viability. In particular, both CY and CY3G induce a dose-dependent decrease in cell proliferation as assessed by the clonogenic test, which is more related to cell proliferation rather than to cell viability.

The measurements of ROS levels, reported in Table 1, indicate a decrease in ROS production after CY, particularly at the lowest concentration; whereas CY3G slightly decreases ROS level only at 25 $\mu\text{mol/l}$. No significant decrease in ROS formation is observed at higher CY3G concentrations.

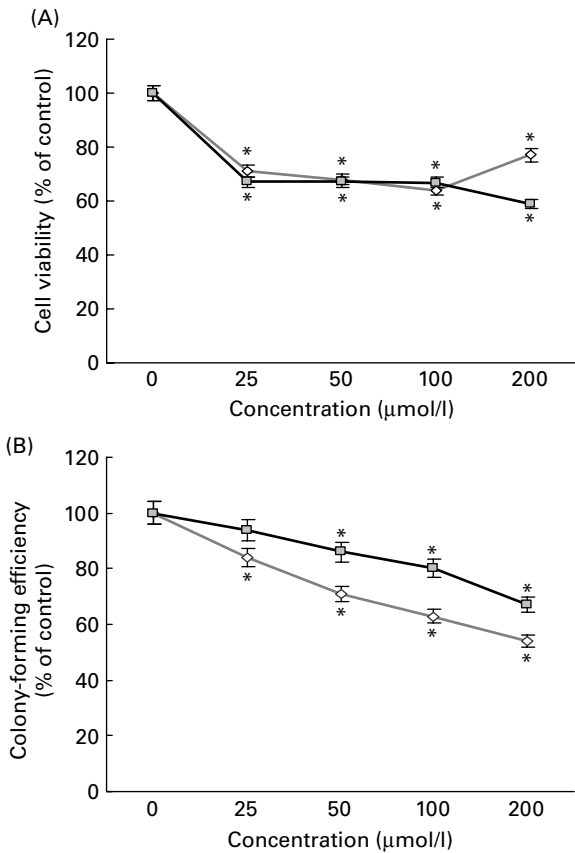


Fig. 1. (A) Cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay) and (B) cell survival (clonogenic test) examined in differently treated human colon carcinoma (CaCo2) cells (◇, cyanidin chloride; □, cyanidin-3-O-β glucopyranoside). The data are expressed as percentage of the control value. Values are means of at least three experiments, each one performed in triplicate (three independent measures), with standard deviations depicted by vertical bars. Mean values were significantly different from those of the control group: **P* < 0.001.

Table 1. Reactive oxygen species analysis in differently treated human colon carcinoma (CaCo2) cells

Treatment (μmol/l)	Fluorescence intensity†	
	Mean	SEM
Control	1461.33	162
CY (25)	624.66*	155
CY (50)	870.49*	110
CY (100)	899.92*	98
CY (200)	877.11*	85
CY3G (25)	1003.60*	92
CY3G (50)	1194.33	108
CY3G (100)	1112.05	91
CY3G (200)	1126.94	110
H ₂ O ₂ (200)‡	2643.66*	155

CY, cyanidin chloride; CY3G, cyanidin-3-O-β glucopyranoside. Mean values were significantly different from those of the control group: **P* < 0.01. †Results are presented as fluorescence intensity normalized for mg protein with each value representing the mean of at least three experiments, each one performed in triplicate (three independent measures). ‡Positive control.

DNA fragmentation of colon carcinoma cells induced by treatment with cyanidin chloride and cyanidin-3-O-β glucopyranoside

The typical Comet assay shows a moderately dose-dependent DNA fragmentation induced on CaCo2 cells after CY exposure at concentrations ranging between 25 and 100 μmol/l (Fig. 2(A)). Conversely, CY3G treatment induces a more dramatic DNA fragmentation at higher concentrations. The present result indicates that CY at low dose is more efficient than CY3G in determining CaCo2 cell DNA damage.

A similar effect is observed when the cellular atypical Comet assay is performed (Fig. 2(B)). This assay is a useful tool to detect any intercalation of a given molecule to DNA. The present results show that the level of DNA fragmentation is clearly increased and that, after the treatment with CY3G, a slightly dose-dependent effect is observed also for the lower concentrations (25 and 50 μmol/l).

However, when the DNA damage is measured by the acellular atypical Comet, which implies the absence of DNA repair systems or any other type of cell protection, DNA fragmentation after CY treatment is more dramatic (Fig. 2(C)). In particular, a dose-dependent effect is exerted by CY within the whole concentration range examined (25–200 μmol/l) while, surprisingly, upon CY3G treatment a significant DNA fragmentation appears only when the highest concentration is used (200 μmol/l).

In order to investigate the capability of CY and CY3G to prevent oxidative induced DNA damage, the atypical Comet assay (cellular and acellular) was performed by challenging agarose-embedded cells or DNA with H₂O₂ either simultaneously or after pretreatment with CY and CY3G (Fig. 3(A), (B)). The results indicate that CY3G is more efficient than CY in counteracting the H₂O₂-induced DNA damage.

Cell cycle-related protein modification after CaCo2 treatment with cyanidin chloride and cyanidin-3-O-β glucopyranoside

To examine molecular events linked to cell cycle progression, the expression of ATM, p53 and p21 as well as of topoisomerase IIβ was analysed by Western blotting. As demonstrated in Fig. 4(A), the treatment with CY induces an increase in ATM expression independently of the concentration of the flavonoid (Fig. 4(A)). On the other hand, a dose-dependent effect is exerted by the treatment with CY3G only at the lowest dose tested (Fig. 4(B)). As regards p53 expression, it is slightly increased by CY independently of the concentration (Fig. 4(A)), whereas the level of the protein is upregulated by CY3G only at 50–200 μmol/l (Fig. 4(B)). CY and CY3G also increase topoisomerase IIβ levels without any dose-dependence, while p21 expression is not modified by the two compounds.

Stress-related proteins

In order to determine the possible involvement of oxidative stress in the chemotherapeutic effects elicited by CY and CY3G on CaCo2 cells, the expression of HSP70 and of OGG1 was measured along with the determination of ROS levels. As evidenced by Western blotting (Fig. 4(A), (B)), the HSP70 level is increased after treatment with the two molecules; differently from the effect of CY3G treatment,

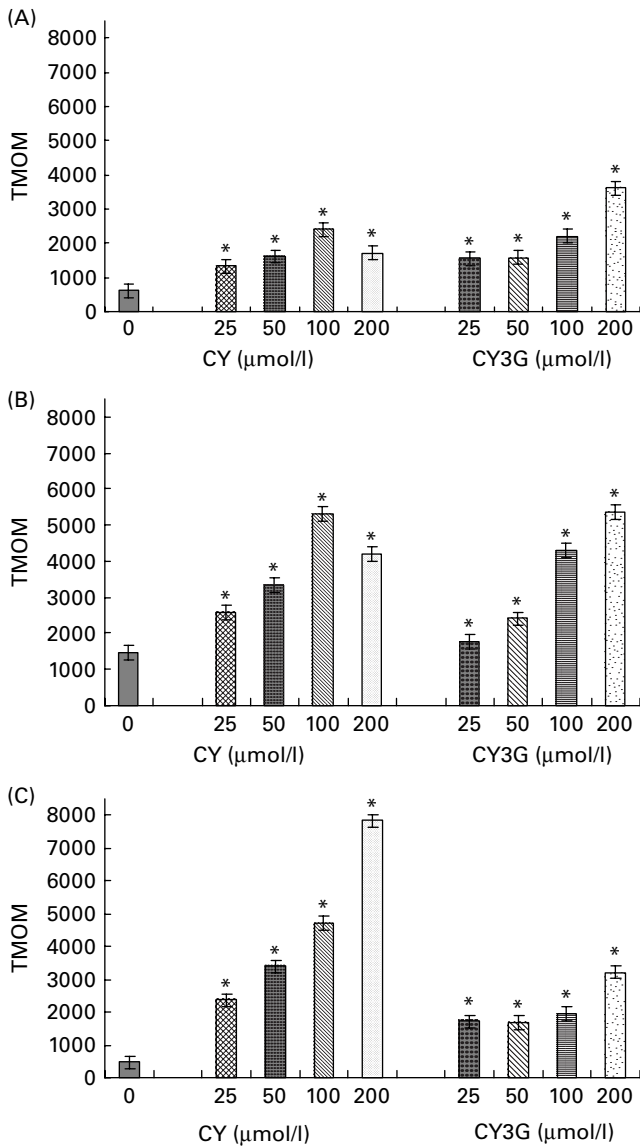


Fig. 2. Comet assay performed in differently treated human colon carcinoma (CaCo2) cells (CY, cyanidin chloride; CY3G, cyanidin-3-O- β glucopyranoside). (A) Typical Comet (cells treated in culture for 24 h, then scraped, embedded in agarose and analysed); (B) atypical cellular Comet (cells treated after being embedded in agarose for 30 min); (C) atypical acellular Comet (treatment performed directly on naked agarose-embedded DNA for 30 min). Values are expressed as tail moment (TMOM), which is the product between the percentage of DNA in the Comet tail (TDNA) and the distance between the head and tail of the Comet (TD). Values are means of three experiments performed in triplicate (three independent measures), with standard deviations depicted by vertical bars. Mean values were significantly different from those of the control group: * $P < 0.001$.

the modulation elicited by CY is slightly dose-dependent. The expression of OGG1 was examined in order to determine the possible relationship with ROS formation and DNA fragmentation. The level of this protein decreases after treatment with 25 $\mu\text{mol/l}$ CY, remains at about the same level as the control after 50 and 100 $\mu\text{mol/l}$ CY, whereas it increases when cells are exposed to 200 $\mu\text{mol/l}$ CY. Conversely, CY3G treatment induces a significant OGG1 increase only at the lower concentration (25 $\mu\text{mol/l}$).

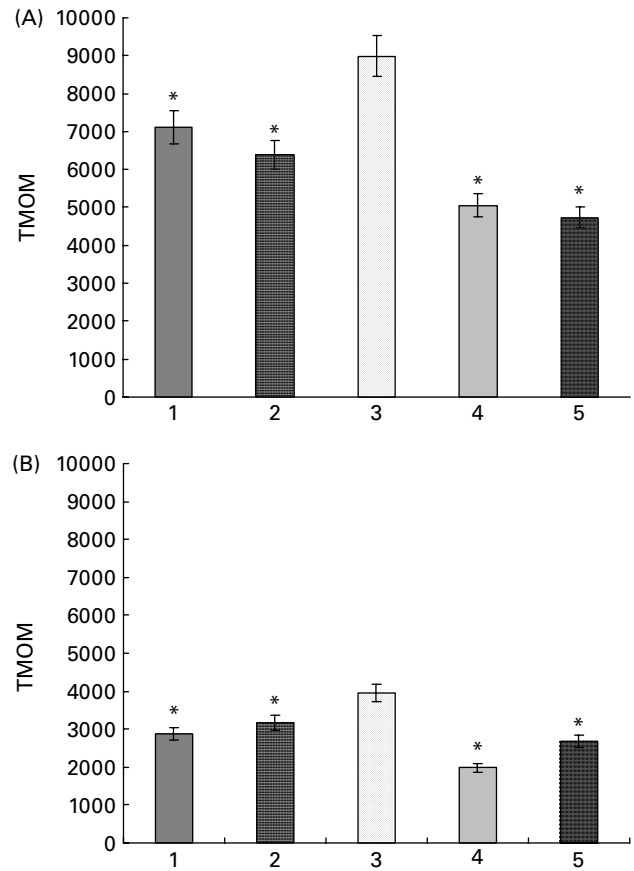


Fig. 3. Atypical Comet assay performed in oxidative stress conditions (200 $\mu\text{mol/l}$ H_2O_2) as: (A) cellular (cells treated after being embedded in agarose); (B) acellular (treatment performed directly on naked agarose-embedded DNA). In both the tested conditions cyanidin chloride (CY) and cyanidin-3-O- β glucopyranoside (CY3G) were used at 50 $\mu\text{mol/l}$ concentration: (1) 50 $\mu\text{mol/l}$ CY and 200 $\mu\text{mol/l}$ H_2O_2 ; (2) 50 $\mu\text{mol/l}$ CY followed by 200 $\mu\text{mol/l}$ H_2O_2 ; (3) 200 $\mu\text{mol/l}$ H_2O_2 ; (4) 50 $\mu\text{mol/l}$ CY3G and 200 $\mu\text{mol/l}$ H_2O_2 ; (5) 50 $\mu\text{mol/l}$ CY3G followed by 200 $\mu\text{mol/l}$ H_2O_2 . Values are expressed as tail moment (TMOM), which is the product between the percentage of DNA in the Comet tail (TDNA) and the distance between the head and tail of the Comet (TD). Values are means of three experiments performed in triplicate (three independent measures), with standard deviations depicted by vertical bars. Mean values were significantly different from those of the 200 $\mu\text{mol/l}$ H_2O_2 -treated cells: * $P < 0.001$.

Discussion

Anthocyanins have been reported to have multiple biological effects including antioxidant and anti-inflammatory activities, inhibition of platelet aggregation and antimicrobial effects⁽²⁾. In the last 10 years, chemotherapeutic effects elicited by anthocyanins in different cancer cells have gained considerable attention^(13,15,19,27,32–34). Nonetheless, the molecular mechanisms related to chemotherapeutic effects of anthocyanins need to be better investigated, also taking into account the concept of ‘persistent oxidative stress in cancer’ formulated by some authors^(35,36).

Extensive *in vitro* and *in vivo* studies have demonstrated the antioxidant and strong free radical-scavenging properties of CY3G and CY. However, literature data are in some cases contradictory in establishing the different properties of the two molecules, due to differences in: (1) chemical structure; (2) applied experimental protocols^(1,2), i.e. the use of anthocyanins

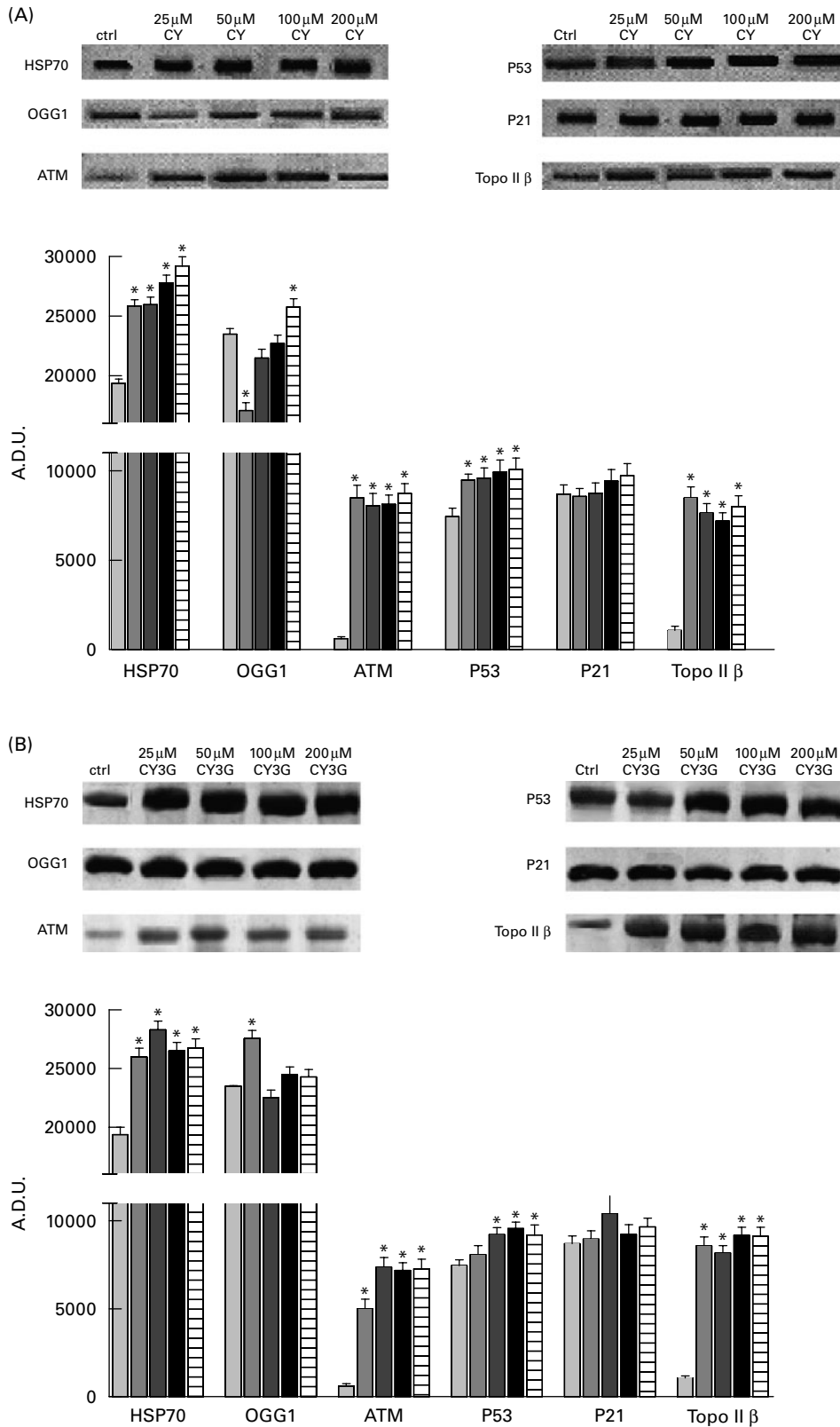


Fig. 4. Immunoblotting analysis performed in differently treated human colon carcinoma (CaCo2) cells. Results, expressed as arbitrary densitometric units (A.D.U.), correspond to the autoradiography densitometric analysis. Representative blots are reported above each graph. Presented is the modulation of protein expression after treatment (24 h) with different concentrations of cyanidin chloride (CY; A) or cyanidin-3-O- β glucopyranoside (CY3G; B): \square , control (CTRL); \blacksquare , 25 μ mol/l; \blacksquare , 50 μ mol/l; \blacksquare , 100 μ mol/l; \blacksquare , 200 μ mol/l. Values are means of three experiments performed in triplicate (three independent measures), with standard deviations depicted by vertical bars. Mean values were significantly different from those of the control group: * $P < 0.001$. ATM, ataxia teleangiectasia mutated protein; HSP70, 70 kDa heat shock protein; OGG1, 8-oxoguanine DNA glycosylase; Topo II β , topoisomerase II β .

as contained in food extracts or as pure chemicals; and (3) absorption and metabolic fate^(2,37).

The present study was designed to investigate possible molecular mechanisms responsible for the anti-carcinogenic power of both CY and CY3G on CaCo2 cells. The two compounds were chosen in order to examine the role of the β -ring. CaCo2 cells were chosen as an appropriate experimental model, since this cell line corresponds to a widely diffuse human tumour and expresses various cytochrome P450 isoforms and Phase II enzymes (such as UDP-glucuronosyltransferase, sulphotransferase and glutathione-S-transferase), which makes CaCo2 cells particularly resistant to toxicants⁽³⁸⁾.

With this respect we studied the effects of CY and CY3G on cell growth/proliferation, modulation of the oxidative status and DNA damage.

We hypothesize that the different results between the clonogenic test and MTT assay could be due to an interference between anthocyanins and the tetrazolium salts utilized for MTT assay. Thus, in our experimental model, the clonogenic test appears to be more reliable. Taken all together the present results demonstrate that CY and CY3G inhibit cell growth as assessed by MTT and clonogenic tests, with the latter showing a dose-dependent inhibition of CaCo2 cell proliferation.

Bearing in mind the concept of 'persistent oxidative stress in cancer' mentioned earlier, it is possible that in the present experimental condition CaCo2 cells survive and continue to grow in the presence of high amounts of H₂O₂ and radical species as observed by different authors in many types of tumour cell^(19,35,36).

Nonetheless, radical species, by activating different signalling transductional/transcriptional pathways or proto-oncogenes (such as MAPkinase, NF- κ B and AP-1), may either sustain the life of tumour cells or cause their demise. Thus, it is reasonable that adequate doses of antioxidant molecules, such as CY/CY3G, could hypothetically promote apoptotic events by: (1) modulating antioxidant defences (i.e. ratio of reduced to oxidized glutathione, haeme oxygenase, etc) and (2) reducing the ability of cancer cells to grow and proliferate.

According to these considerations, the results of ROS analysis performed by 2',7'-dichlorodihydrofluorescein assay (Table 1) indicate that the antiproliferative/cytotoxic activity exhibited by CY was sustained by decreased endocellular ROS level and the related signalling events. Diversely, CY3G exhibits antiproliferative/cytotoxic activity only at the lowest concentration, indicating the involvement of different intracellular pathways.

To better define a possible correlation between cell proliferation, ROS level and chemotherapeutic potential of CY and CY3G, we focused our attention on the analysis of the treatment-induced DNA damage by single-cell gel electrophoresis (Comet assay). Taking into account that tail intensity (TDNA) indicates the number of DNA breaks, and that it is commonly assumed that the tail intensity, rather than the tail length⁽³⁹⁾, increases beyond some critical amount of damage, we consider TMOM as a more comprehensive parameter of DNA damage.

As evidenced by the present results, both the anthocyanins induce DNA fragmentation, particularly at the highest concentration (Fig. 2(A)) and when the damage is measured by atypical cellular Comet assay (Fig. 2(B)). The higher damage evidenced by this version of the Comet assay can be explained

by considering a lower efficiency of the DNA repair systems in the agarose-embedded cells with respect to cells in culture. Interestingly, all Comet images showed a fragmentation shape known as 'hedgehog', considered by some authors as a hallmark of apoptotic DNA damage⁽⁴⁰⁾. Moreover, the atypical acellular Comet (performed after cell lysis, on naked DNA) let us evidence a possible direct interaction between CY and DNA (Fig. 2(C)). As regards the effect exerted by CY3G, two possible explanations may be given: (1) the DNA damage observed through typical and atypical cellular Comet (Fig. 2(A), (B)) could be mostly due to its metabolites, this could be the reason why the damage is reduced when its direct interaction with DNA is considered (atypical acellular Comet; Fig. 2(C)); and (2) CY3G could form DNA cross-links or DNA adducts, thus decreasing tail length and giving rise to a 'falsely' reduced damage. These hypotheses are supported by literature data demonstrating the polyphenols' DNA intercalating capability with consequent induction of DNA strand breaks⁽⁴¹⁾. In addition, some preliminary data obtained in our laboratory (data not shown) evidence a shift towards lower energies (higher wavelengths) both in UV and in visible, contextually with a reduction of the extinction coefficient, when a DNA titration with CY or CY3G is carried out (ratio DNA:flavonoid 1:0.2–1:3); an observation reinforcing the hypothesis of a substantial affinity between CY/CY3G and DNA.

To acquire more information on the possible anthocyanins–DNA interaction and/or on the antioxidant capability of the molecules, we also performed the cellular and acellular atypical Comet assay on CaCo2 cells treated with CY/CY3G and H₂O₂ (Fig. 3(A), (B)), a molecule well known either to promote or inhibit the proliferation of cancer cells^(19,36,42). H₂O₂ treatment (200 μ mol/l) was performed either simultaneously or after CY/CY3G pretreatment (50 μ mol/l). The TMOM values of the cells treated with CY/CY3G plus H₂O₂ were compared with those of the cells treated with H₂O₂ alone.

The CY/CY3G TMOM values measured by the cellular atypical Comet (Fig. 3(A)) are higher than those obtained by the acellular version of the assay (Fig. 3(B)), probably because if the treatments are performed in the presence of the whole cells, H₂O₂ metabolization occurs and increases DNA fragmentation. As reported in Fig. 3(A), (B)), CY3G seems to be more effective than CY in counteracting H₂O₂-induced DNA damage. However, in order to establish if CY3G is a more efficient 'antioxidant' than CY, further investigations are in progress in our laboratory by the employment of a polarized light assay and other versions of the Comet assay.

In the present study we also examined the expression level of four cell cycle progression-involved proteins (topoisomerase II β , p53, p21 and ATM) and two oxidative stress-related proteins (HSP70 and OGG1). Topoisomerase II β is a nuclear enzyme which induces transient DNA strand breaks to solve topological problems of DNA replication and gene transcription; p53, mutated in about 50% of cancer types, is a sentry protein present at very low level in resting cells and in some systems co-operates with p21 in controlling cell cycle progression. ATM, an activator of p53, works in resting non-proliferating cells⁽⁴³⁾. The present findings suggest that an increase in ATM triggers p53 expression that could be mainly represented by the mutated form, unable to act as a transcription factor for p21⁽⁴⁴⁾. The relevant increase in

topoisomerase II β level could be triggered as a reaction to the well-known topoisomerase poisoning effect exerted by anthocyanins⁽⁴¹⁾. This effect is a decrease of the DNA protective action elicited by topoisomerase I and II (and β).

Interestingly, for the first time to our knowledge, we examined the modulation of the HSP70 and OGG1 expression levels after treatment with anthocyanins. HSP70, belonging to the family of the highly conserved heat shock proteins, is well known to exert an important action in biotic and abiotic stress conditions as well as in cancerogenesis^(24,26). OGG1, constitutively expressed in both cancerous and non-cancerous human cells, is the only protein able to remove from DNA 7,8-dihydro-8-oxoguanine, an abundant mutagenic lesion caused by exposure to free radicals and ROS^(22,23). It must be underlined that in the CaCo2 cells the HSP70 basal level appears to be very high, thus suggesting the pivotal role of heat shock proteins in carcinogenesis. So the observed HSP70 increase after CY/CY3G treatment may be considered as a cellular attempt to prevent apoptotic/necrotic cell death. On the other hand, the different ability of CY and CY3G in modulating OGG1 expression can be explained by taking into consideration both the presence of DNA lesions generated by oxidative stress and the level of tumorigenicity.

In summary, CY seems to act on CaCo2 cells in a ROS-dependent way, probably through a direct interaction with DNA (see atypical Comet assay results). Furthermore, CY induces an increase in a ATM, p53, topoisomerase II and in HSP70 (the latter being somehow dose-dependent), without modulating p21. The up-regulation of HSP70 expression may be considered as a response of the cancer cells to the insult induced by the dose-dependent decrease in ROS level (Table 1).

Conversely, CY3G appears to act in a ROS-independent way, since it does not modulate ROS levels. However, its effect on the decrease in DNA fragmentation induced by H₂O₂ highlights an important antioxidant ability. This effect could be favoured either by its DNA-stabilizing cross-linking capability⁽²⁰⁾ or by the presence of the sugar moiety. As regards the expression of ATM, topoisomerase II, p53 and p21, CY3G shows almost the same behaviour as CY; the only difference being a completely non-dose-dependent increase in HSP70.

In conclusion, the present results indicate that the substitution pattern at the β -ring could interfere with the anthocyanin-modulated signalling cascades involved in the regulation of CaCo2 cell growth. In addition, in the present experimental conditions, CY and CY3G exhibited remarkable biological properties, this substantiating a possible new chemotherapeutic role for this class of molecules. Finally, the present data encourage the use of an atypical Comet assay as a useful tool in assessing DNA–molecule interactions.

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