British Journal of Nutrition (2005), **94**, 211–220
DOI: 10.1079/BJN20051425

Cyanidin-3-*O*-β-glucopyranoside, a natural free-radical scavenger against aflatoxin B1- and ochratoxin A-induced cell damage in a human hepatoma cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2)

M. C. Guerra[1]*, F. Galvano[2], L. Bonsi[3], E. Speroni[1], S. Costa[1], C. Renzulli[1] and R. Cervellati[4]

[1]*Department of Pharmacology, University of Bologna, Via Irnerio 48, 40 126 Bologna, Italy*
[2]*Department of Agro-forestry, Environmental Science and Technology, University of Reggio Calabria, Piazza San Francesco 7, Reggio Calabria, Italy*
[3]*Institute of Histology and General Embryology, University of Bologna, Via Belmeloro 8, Bologna, Italy*
[4]*Department of Chemistry 'G. Ciamician', University of Bologna, Via Selmi 2, Bologna, Italy*

(Received 22 June 2004 – Revised 20 December 2004 – Accepted 11 January 2005)

Recent findings have suggested that oxidative damage might contribute to the cytotoxicity and carcinogenicity of aflatoxin B1 (AFB1). Induction of oxidative stress also plays an important role in the toxicity of another mycotoxin, ochratoxin A (OTA). In the present study, the protective effect of cyanidin-3-*O*-β-glucopyranoside (C-3-G; an anthocyanin contained in oranges, blackberries, strawberries and cranberries) against AFB1- and OTA-induced cytotoxicity was investigated in a human hepatoma-derived cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). The ability of C-3-G to reduce the production of reactive oxygen species (ROS), the inhibition of protein and DNA synthesis and the apoptosis caused by the two mycotoxins was also investigated in both cell lines. Our experiments proved the significant cytoprotective effect of C-3-G *in vitro* against OTA- and AFB1-induced cell damage. In particular, 24 h of pretreatment with 50 μM-C-3-G inhibited the cytotoxicity of 10 μM-AFB1 (by 35 %) and of 10 μM-OTA (by 25 %) in Hep G2 cells ($P<0{\cdot}001$) and of 10 μM-AFB1 (by 10 %, $P<0{\cdot}01$) and of 10 μM-OTA (by 14 %, $P<0{\cdot}05$) in CaCo-2 cells. Moreover, 50 μM-C-3-G attenuated ROS production induced by the two toxins in both cell lines ($P<0{\cdot}05$). Inhibition of DNA and protein synthesis induced by the mycotoxins was counteracted by pretreatment with the antioxidant at 50 μM. Similarly, apoptotic cell death was prevented as demonstrated by a reduction of DNA fragmentation and inhibition of caspase-3 activation. The *in vitro* free-radical scavenging capacity of the anthocyanin was tested with the Briggs–Rauscher oscillating reaction. This system works at pH approximately 2. The results showed good scavenging power, in accordance with the observed inhibition of ROS production.

Mycotoxins: Cyanidin-3-*O*-β-glucopyranoside: Hep G2 cells: CaCo-2 cells

Mycotoxins are fungal secondary metabolites, produced by many species of *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps* and *Alternaria* (Bennett & Keller, 1997) and known to be associated with human and animal disease. Among the most common mycotoxins, distributed world-wide, are aflatoxins, ochratoxin A (OTA), trichothecenes, zearalenone and fumonisins. Their presence in food and feeds can present a danger to animal and human health, since the consumption of a mycotoxin-contaminated diet may induce acute and chronic effects resulting in a teratogenic, carcinogenic (mainly for liver and kidney), oestrogenic or immunosuppressive impact on animals and man. Animals usually suffer due to use of lower quality feeds (Steyn & Stander, 1999).

Aflatoxins and ochratoxins are structurally different mycotoxins, reported to be involved in the development of human cancers (International Agency for Research on Cancer, 1993). Aflatoxin B1 (AFB1) is one of the most potent naturally occurring genotoxic and hepatocarcinogenic agents (Massey *et al.* 1995). Although its main mechanism of action involves the formation of DNA adducts by the ultimate carcinogen of AFB1 (AFB-8,9-exoepoxide), important findings have suggested that oxidative damage might contribute to the cytotoxicity and carcinogenicity of AFB1 (Amstad *et al.* 1984). OTA, *N*-[[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]carbonyl]-3-phenyl-L-alanine, is a nephrotoxic, hepatotoxic and teratogenic mycotoxin produced by storage moulds (mainly by species of *Aspergillus* and *Penicillium*; Kuiper-Goodman & Scott, 1989). The mechanism of OTA carcinogenicity has not been definitively elucidated. It is not clear whether OTA acts as a genotoxic carcinogen or whether its carcinogenicity is due to an indirect mechanism such as induction of cytotoxicity and oxidative damage. It has been shown that OTA induces oxidative stress when administered to rats or added to liver or kidney microsomes (Rahimtula *et al.* 1988). The generation of reactive oxygen species (ROS) may be mediated via the formation of an Fe^{3+}–OTA complex (Omar & Rahimtula, 1993).

Recent studies have documented the ability of naturally occurring chemicals with antioxidant properties to prevent the oxidative effects of mycotoxins. Much less information is available

Abbreviations: AFB1, aflatoxin B1; BR, Briggs–Rauscher; C-3-G, cyanidin-3-*O*-β-glucopyranoside; H_2DCF-DA, dihydrodichlorofluorescein diacetate; NR, neutral red; OTA, ochratoxin A; rac, relative activity with respect to concentration; ROS, reactive oxygen species.

* **Corresponding author:** Professor Maria Clelia Guerra, fax +39 51 248862, email mcguerra@biocfarm.unibo.it

from studies carried out using dietary antioxidants and mycotoxins (Rizzo *et al.* 1994; Abel & Gelderblom, 1998; Yin *et al.* 1998; Belmadani *et al.* 1999; Galvano *et al.* 2001; Gautier *et al.* 2001). The protective properties of antioxidants probably result from their ability to act as superoxide anion scavengers, thereby protecting cell membranes from mycotoxin-induced damage; in some cases, antioxidant vitamins may play a role in preventing mycotoxicosis (Atroshi *et al.* 1987).

Anthocyanins, secondary metabolites present in fruits and vegetables and responsible for their pigmentation, have recently gained increasing interest as strong antioxidants with the potential to prevent oxidative damage caused by ROS and consequently protect against some cancers and CVD (Vinson *et al.* 1995; Pietta, 2000; Wang & Stretch, 2001). Among anthocyanins, cyanidin-3-*O*-β-glucopyranoside (C-3-G), contained in pigmented oranges and fruits of the berry family, has been found to possess good antioxidant power owing to the presence of several phenolic hydroxyl groups (Fig. 1). C-3-G is a polyphenol and glycosylation, which occurs on specific carbon atoms of the molecule, increases its stability.

In the present study we investigated the ability of C-3-G to inhibit AFB1- and OTA-induced cytotoxicity in a human hepatoma-derived cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). The ability of C-3-G to prevent ROS production, DNA and protein synthesis inhibition and apoptotic cell death, induced by OTA and AFB1, were also considered. The antioxidant potential of C-3-G was determined by a new method based on the inhibitory effects of antioxidants on oscillations of a hydrogen peroxide–acidic iodate–malonic acid–Mn(II)-catalysed system (Cervellati *et al.* 2001).

Experimental methods

Chemicals

C-3-G (>99 %) was obtained from Apin Chemicals Ltd (Abingdon, UK). AFB1 and OTA (>99·5 %) were from Sigma (St. Louis, MO, USA). Iscove-modified Dulbecco's medium, fetal bovine serum and penicillin–streptomycin were from Bio-Whittaker (Walkersville, MD, USA). Malonic acid (reagent grade, >99 %), $MnSO_4 \cdot H_2O$ (reagent grade, >99 %) and $NaIO_3$ (reagent grade, >99·5 %) were all procured from Merck. Milan, Italy $HClO_4$, H_2O_2 and all other chemicals were of analytical grade. All stock solutions were prepared from doubly distilled water.

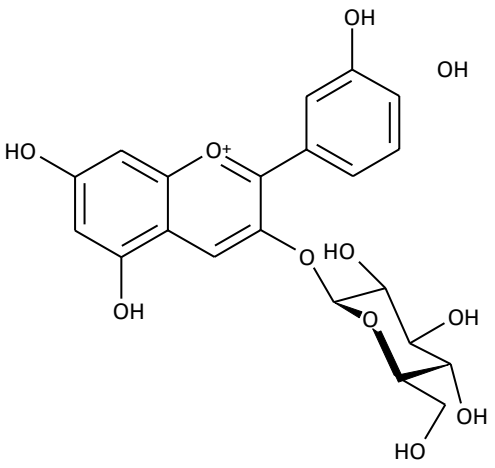

Fig. 1. The structure of cyanidin-3-*O*-β-glucopyranoside.

Cell culture

Intestinal CaCo-2 cells and Hep G2, derived from a human hepatoblastoma, were obtained from American Type Culture Collection (Manassas, VA, USA). Hep G2 cells were cultured in Iscove-modified Dulbecco's medium supplemented with 10 % fetal calf serum and 1 % penicillin–streptomycin (all v/v). CaCo-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 1 % penicillin–streptomycin and 2 mM-glutamine. All cells were maintained in an atmosphere of 5 % CO_2 and at 37°C.

Media were replenished every 2 d for both cell types, and the cells harvested and passaged for exposure to the test agents while in the logarithmic growth phase.

Determination of cell viability

Cytotoxicity of OTA and AFB1 (dissolved respectively in methanol and dimethyl sulfoxide) towards Hep G2 and CaCo-2 cells was evaluated by the neutral red (NR) assay. Solvent controls (denoted as 0 μM) were 0·025 % dimethyl sulfoxide (AFB1 solvent) and 0·07 % methanol (OTA solvent). Cytotoxicity was determined by quantifying the ability of cells to incorporate NR into their lysosomes, as a decrease in NR dye uptake is indicative of injured or growth-inhibited cells (Gramenitsky, 1963). Cells were seeded at 10 000 cells/well in 96-well plates. The following day, cells were treated with OTA or AFB1, at concentration of 0–100 μM, for 24, 48 or 72 h. Also, C-3-G was tested in a wide range of concentrations (0–250 μM), for 24 h, in order to verify the possible effects on cell viability (data not shown).

To evaluate the protective capacity of C-3-G against AFB1- or OTA-induced cytotoxicity, cells were pre-treated with the antioxidant (25 and 50 μM) for 24 h, and then the medium was replaced with new medium containing OTA or AFB1 at a concentration of 2·5, 5 or 10 μM for 48 h. After the treatment, cells were washed with PBS and incubated with NR (50 μg/ml) in growth medium for 4 h. The NR medium was removed, the cells washed with PBS, and elution buffer (50 % ethanol, 1 % acetic acid) was added to release the dye. Subsequently, absorbance readings were collected at 540 nm using a Multiskan plate reader (Spectra Classic; Tecan GmbH, Crailsheim, Germany). Cell survival was expressed as a percentage of solvent-exposed control survival and IC_{50} values (concentration that inhibits 50 % of cell survival) were calculated for the toxins alone at the different time points.

Determination of production of reactive oxygen species

Hep G2 and CaCo-2 cells were seeded in 96-well culture plates at 1×10^4 cells/well and allowed to grow for 24 h. Cells were then incubated for 30 min at 37°C in serum-free medium containing 20 μM-dihydrodichlorofluorescein diacetate (H_2DCF-DA). After aspiration of H_2DCF-DA, the cells were rinsed once and subsequently exposed to increasing concentrations of OTA (0–100 μM) or AFB1 (0–100 μM) in serum- and phenol red-free medium. ROS production was measured after 24 h incubation on a Cytofluor 2300 Fluorescence Measurement System (Millipore, Bedford, MA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In experiments

with the antioxidant, cells were incubated 24 h after plating with two concentrations of C-3-G (25 or 50 μM). Following the 24 h pre-incubation period, the cells were treated with H_2DCF-DA as described earlier. After removal of H_2DCF-DA, cells were exposed to a fixed concentration of OTA (25 μM) or AFB1 (10 μM). ROS levels were determined 24 h later as described earlier. Relative ROS production (percentage of the control) was expressed as the ratio of the fluorescence of the treated sample over the response in the appropriate control [(fluorescence$_{treatment}$/fluorescence$_{control}$) × 100].

Cellular synthesis of protein and DNA

Hep G2 and CaCo-2 cells were cultured in 24-well multidishes for 24 h at 37°C as described earlier. After 24 h of pre-treatment with C-3-G, the medium was replaced with new medium containing AFB1 or OTA at 2·5, 5 or 10 μM for 24 h. Control cultures were prepared by adding 0·025 % dimethyl sulfoxide (AFB1 solvent) and 0·07 % methanol (OTA solvent). The effects of C-3-G alone, at 25 and 50 μM, on protein and DNA synthesis were also evaluated after a 24 h pre-incubation.

After 24 h of incubation, 0·037 MBq of the radioactive protein synthesis precursor [^{3}H]leucine (specific activity, 185 GBq/mmol) or 0·037 MBq of the radioactive DNA synthesis precursor [^{3}H]thymidine (specific activity, 3250 GBq/mmol) was added. After further incubation for 3 h, the cells were treated as described by Cochereau *et al.* (1997) to determine the inhibition rate of protein and DNA synthesis by the measurement of radioactivity with a beta counter. All results, calculated as dpm/mg protein, are expressed as a percentage of protein or DNA synthesis inhibition with respect to the control.

Determination of protein concentration was performed using Bradford's colorimetric method (Bradford, 1976).

Assays of apoptosis

DNA fragmentation assay. Hep G2 and CaCo-2 cell culture and treatment were performed as described in the previous section. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and suspended in PCR-grade H_2O to detect DNA fragmentation using the ApoAlert™ LM-PCR Ladder Assay Kit (Clontech, Mountain View, CA, USA). The genomic DNA was mixed with ligation mix (approximately 70 μl ligation mix per 1 μg genomic DNA), which was heated to 55°C for 10 min and then cooled to 10°C over approximately 1 h to allow the adaptor oligonucleotides to anneal, next 0·5 μl T4 DNA ligase was added and the solution was kept at 16°C for 12–16 h. The adaptor-ligated DNA were used as templates for PCR under the following conditions: thirty cycles of 72°C for 8 min, 94°C for 1 min and 72°C for 3 min; then 72°C for 15 min. The PCR mixture comprised 10 × LM-PCR mix, adaptor-ligated DNA, PCR-grade H_2O and 50 × advantage cDNA polymerase mix. Finally, 10 μl of each reaction was processed on a 4 % agarose/ethidium bromide gel at 6 V/cm for approximately 2·5 h.

Caspase-3 colorimetric assay. Caspase-3 colorimetric assay (R & D systems, Minneapolis, MN, USA) was used to determine the increased enzymatic activity of caspase-3 protease in apoptotic Hep G2 and CaCo-2 cells following incubation with OTA or AFB1, with or without C-3-G pretreatment. Also, the antioxidant alone was tested to verify an effect on caspase-3 activity at the concentration used in this study.

This test is based on the addition of a caspase-specific peptide conjugated to a reporter molecule, *p*-nitroanilide. Cleavage of the peptide by caspase releases the chromophore *p*-nitroanilide, which is quantified spectrophotometrically at 405 nm. Briefly, cells were cultured with AFB1 or OTA for 24 h, with or without the C-3-G pretreatment, and caspase-3 activity was measured as per the manufacturer's recommendations, expressing the results as fold increase in caspase-3 activity of apoptotic cells relative to uninduced cells.

Antioxidant activity test

Antioxidant activity of C-3-G was measured using the chemical *in vitro* method reported by Cervellati *et al.* (2001), which is based on the inhibitory effects of ROS scavengers on the oscillations of the Briggs–Rauscher (BR) reaction. The BR system (Briggs & Rauscher, 1973) consists of hydrogen peroxide, acidic iodate, malonic acid and Mn(II) as catalyst and works at pH of approximately 2, similar to that in the human stomach. Like other methods, the BR reaction is based on the generation of free radicals in the reaction mixture. The generated hydroperoxyl radicals ($HOO^{\bullet}$) are among the main intermediates of the BR system. The mechanism of the action of antioxidants against $HOO^{\bullet}$ radicals in the BR system has been described in detail elsewhere (Cervellati *et al.* 2001, 2004*a*). In brief, when antioxidant scavengers of free radicals are added to an active oscillating BR mixture there is an immediate quenching of the oscillations, an inhibition time that linearly depends on the concentration of the antioxidant added, and a subsequent regeneration of the oscillations. Oscillations are followed potentiometrically using a bright Pt electrode–reference electrode couple. Relative antioxidant activities with respect to a substance chosen as a standard are determined on the basis of the inhibition times. The BR xreaction method was successfully tested on several white wines (Höner *et al.* 2002), fruit and vegetable extracts (Höner & Cervellati, 2002), extracts of *Cynara scolymus* (Speroni *et al.* 2003) and polyphenols from *Polygala* spp. (Cervellati *et al.* 2004*b*) and from *Wulfenia carinthiaca* Jacq. (Cervellati *et al.* 2004*c*).

BR mixtures were prepared by mixing the appropriate amounts of stock solutions of reagents using pipettes or burettes in a 100 ml beaker to a total volume of 30 ml. The order of addition was malonic acid, $MnSO_4$, $HClO_4$, $NaIO_3$ and H_2O_2. Oscillations start after the addition of H_2O_2. Then 1·0 ml of a suitably diluted aqueous solution of sample was added to the active oscillating BR mixture after the third oscillation and the inhibition time measured.

The standard chosen was resorcinol (Cervellati *et al.* 2001). The straight line for this 'standard' is:

$$t_{inhib}(s) = 465{\cdot}1(\mu M^{-1}\,s) \times \mathrm{conc}(\mu M)\text{-}1181(s) \quad (R^2 = 0{\cdot}994).$$

The antioxidant activity relative to this standard was then calculated as the ratio:

$$\mathrm{rac} = \mathrm{conc\ (std)}/\mathrm{conc\ (smp)},$$

where rac is the relative activity with respect to concentration (Cervellati *et al.* 2001), conc (std) and conc (smp) are the concentrations (in μM) of the standard and the sample that give the same inhibition time.

Statistical analysis

Significance of differences between different data groups was first determined using a one-way ANOVA and followed by the Newman–Keuls multiple comparison test, using Prism 3·0 (Graph Pad Software Inc.; www.graphpad.com). Differences were considered significant if $P<0·05$.

Results

Influence of cyanidin-3-O-β-glucopyranoside on ochratoxin A- and aflatoxin B1-induced loss of cell viability

The viability of Hep G2 cells incubated for 24, 48 and 72 h, and of CaCo-2 cells incubated for 24 and 48 h, decreased in a concentration-dependent manner with increasing OTA (0–250 μM), as assessed by the NR assay (Fig. 2(A and C)). Loss of cell viability was also time-dependent: viability after exposure to OTA for 48 h (up to 80 % mortality, 25 μM) and 72 h (up to 90 % mortality, 25 μM) was significantly ($P < 0·05$ for both 48 and 72 h treatments) lower than that after 24 h incubation (51·4% mortality, 25 μM) in Hep G2 cells; the same can be said for CaCo-2 cells (IC_{50} for 24, 48 and 72 h are respectively 160, 10 and 7·4 μM). An analogous concentration–time-dependent cytotoxicity (IC_{50}: respectively 7, 2 and 0·5 μM after 24, 48 and 72 h in Hep G2 cells; 10, 2 and 0·75 μM in CaCo-2 cells) was also observed after AFB1 (0–100 μM) exposure (Fig. 2(B and D)).

After assessing the sensitivity of Hep G2 and CaCo-2 cells to the two mycotoxins, both cell lines were pre-incubated for 24 h with 25 or 50 μM C-3-G and subsequently challenged with OTA or AFB1 (2·5, 5 or 10 μM) in the absence of C-3-G for 48 h. Pretreatment with C-3-G led to a clear, dose-dependent protective effect on cell toxicity, reducing both OTA- and AFB1-induced cell mortality (Fig. 3). In particular, 24 h of pretreatment with 50 μM-C-3-G restored cell viability by up to 34 % ($P<0·001$) in Hep G2 and up to 10 % ($P<0·05$) in CaCo-2 with respect to 10 μM-AFB1 treatment alone, and by up to 20 % ($P<0·001$) in Hep G2 and up to 23 % ($P<0·01$) in CaCo-2 with respect to 10 μM-OTA treatment alone. The protective effect of C-3-G was more pronounced at the higher concentration and in the Hep G2 cell line.

When tested, no effects of C-3-G alone were observed on Hep G2 and CaCo-2 viability at the concentrations used in the present study (data not shown).

Influence of cyanidin-3-O-β-glucopyranoside on production of reactive oxygen species

After 24 h incubation with increasing concentration of OTA or AFB1 (0–100 μM), there was a dose-dependent ROS production in both Hep G2 and CaCo-2 cells (data not shown). A single dose of OTA (25 μM) and AFB1 (10 μM), able to induce a significant ($P<0·05$) increase in ROS levels above those in solvent-treated controls (methanol and dimethyl sulfoxide), was used to test the ability of C-3-G to reduce oxidative stress. Cells were pre-incubated for 24 h with C-3-G (25 or 50 μM) and subsequently challenged with OTA or AFB1. Treatment with the antioxidant (50 μM) significantly decreased the OTA- and AFB1-induced ROS response (Fig. 4). In detail, C-3-G achieved reductions in ROS production of 8 % and 11 % in Hep G2 treated with 10 μM-AFB1 and 25 μM-OTA, respectively ($P<0·05$), and of 12 % in CaCo-2 cells treated with 25 μM-OTA ($P<0·05$). The lowest concentration of C-3-G (25 μM) failed to reduce ROS levels in a significant manner.

Influence of cyanidin-3-O-β-glucopyranoside on total DNA and protein synthesis reduction induced by aflatoxin B1 and ochratoxin A

After 24 h of incubation with the two mycotoxins, [^{3}H]thymidine and [^{3}H]leucine incorporation was used to analyse the effect of OTA and AFB1 on DNA and protein synthesis. Treatment with

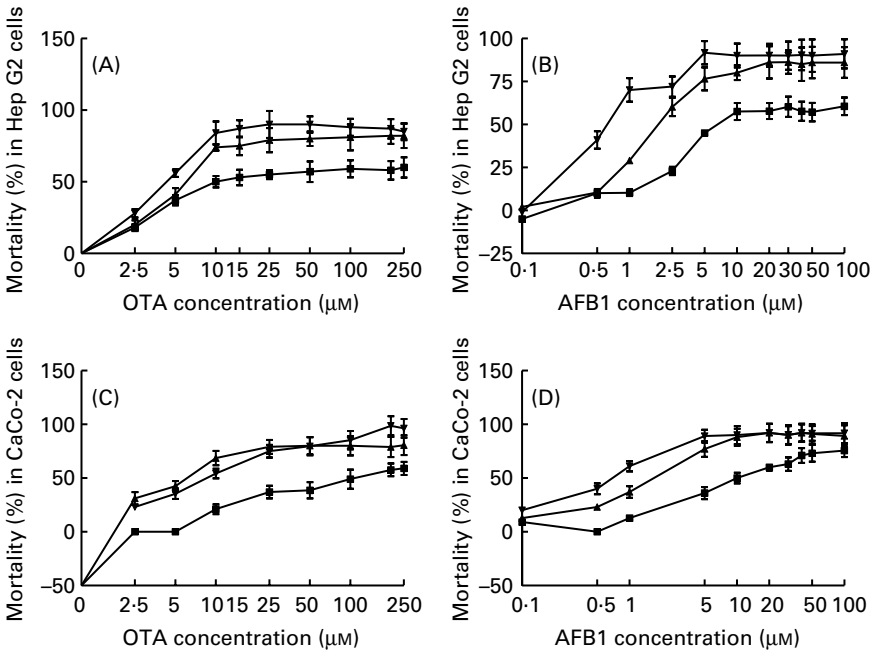

Fig. 2. Cytotoxicity induced by (A) ochratoxin A (OTA; 0–250 μM) and (B) aflatoxin B1 (AFB1; 0–100 μM) in a human hepatoma-derived cell line (Hep G2 cells), and (C) OTA (0–250 μM) and (D) AFB1 (0–100 μM) in a human colonic adenocarcinoma cell line (CaCo-2 cells) after incubation for 24 h (■), 48 h (▲) and 72 h (▼), as assessed by the neutral red assay. Values are means, with their standard deviation shown as vertical bars, of three independent experiments.

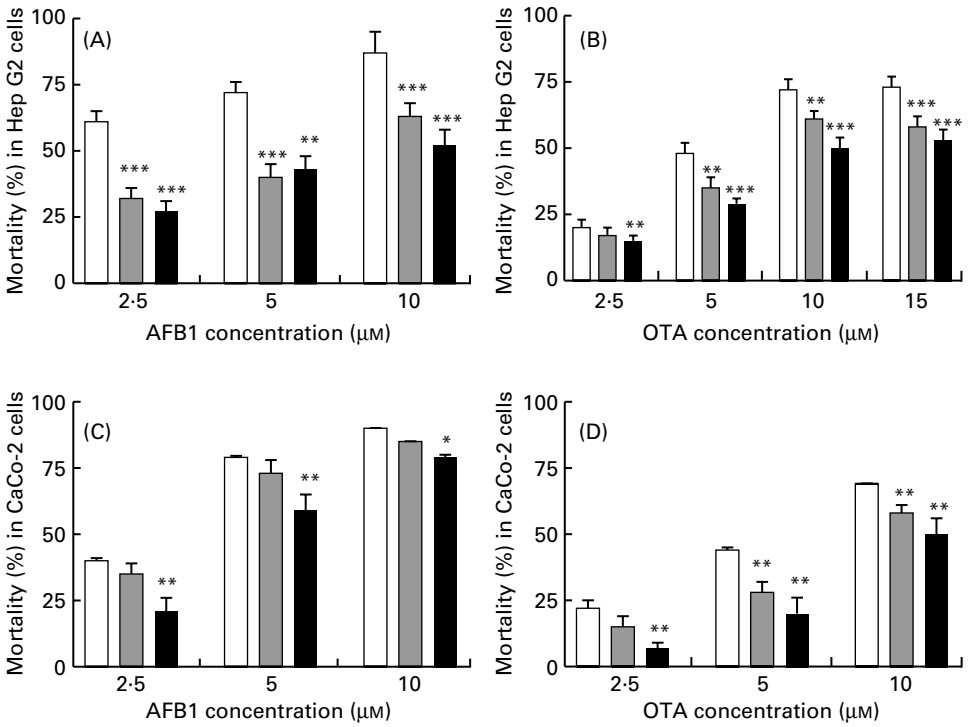

Fig. 3. Influence of 24 h pre-incubation with various concentrations (□, 0 μM (control); ▨, 25 μM; ■, 50 μM) of cyanidin-3-*O*-β-glucopyranoside on cell mortality induced by 48 h incubation with mycotoxins, as assessed by the neutral red assay. (A) Aflatoxin B1 (AFB1; 2·5, 5 and 10 μM)- and (B) ochratoxin A (OTA; 2·5, 5, 10 and 15 μM)-induced cell mortality in a human hepatoma-derived cell line (Hep G2 cells), and (C) AFB1 (2·5, 5 and 10 μM)- and (D) OTA (2·5, 5 and 10 μM)-induced cell mortality in a human colonic adenocarcinoma cell line (CaCo-2 cells). Values are means, with their standard deviation shown as vertical bars, of three independent experiments. Mean values were significantly different from those of the control group (toxin alone): *$P<0·05$, **$P<0·01$, ***$P<0·001$ (one-way ANOVA followed by Newman–Keuls multiple comparison test).

increasing concentrations of OTA and AFB1 had a dose-dependent influence on basal [^{3}H]thymidine and [^{3}H]leucine incorporation, which were decreased by up to 80 % and 70 %, respectively, in Hep G2 and up to 80 % and 25 %, respectively, in CaCo-2 with 10 μM-AFB1; and by up to 55 % and 60 %, respectively, in Hep G2 and up to 80 % and 55 %, respectively, in CaCo-2 with 10 μM-OTA.

Pre-incubation of Hep G2 cells with 50 μM-C-3-G led to a significant increase of [^{3}H]thymidine incorporation ($P<0·001$) after treatment with AFB1 2·5 μM (by 20 %) and 5 μM (by 25 %) compared with the toxin alone (Fig. 5(A)). Moreover, the inhibition of protein synthesis induced by 2·5, 5 and 10 μM-AFB1 (by 15, 15 and 25 %, respectively) was significantly reduced by 50 μM-C-3-G ($P<0·001$; Fig. 5(B)).

Treatment with 25 μM-C-3-G had qualitatively similar but quantitatively reduced effects on both protein and total DNA synthesis inhibition. Similarly, an evident recovery ($P<0·001$ and $P<0·01$) of total DNA and protein synthesis, strongly depressed by OTA, was achieved by 24 h exposure to C-3-G at the higher concentration (50 μM; Fig. 5(C and D)).

In CaCo-2 cells, pre–incubation with 50 μM-C-3-G significantly reduced ($P<0·001$) the inhibition of DNA and protein

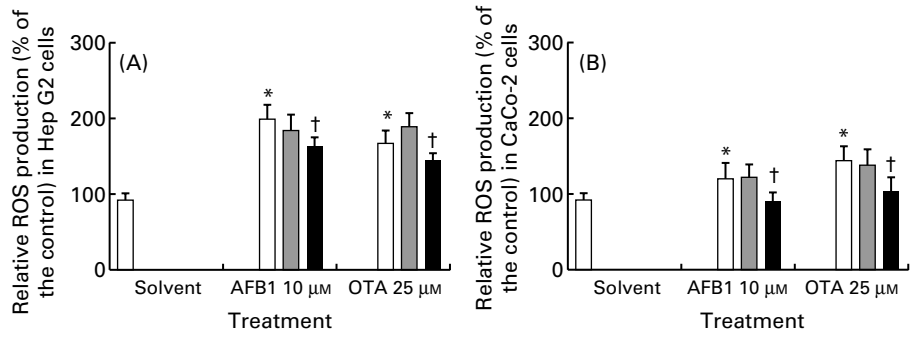

Fig. 4. The effect of cyanidin-3-*O*-β-glucopyranoside (C-3-G) on ochratoxin A (OTA)- and aflatoxin B1 (AFB1)-induced production of reactive oxygen species (ROS) in (A) a human hepatoma-derived cell line (Hep G2 cells) and (B) a human colonic adenocarcinoma cell line (CaCo-2 cells). Cells were pre-incubated for 24 h with C-3-G (□, 0 μM; ▨, 25 μM; ■, 50 μM). Then OTA (25 μM) or AFB1 (10 μM) was added and the ROS response was measured 24 h later. Values are means, with their standard deviation shown as vertical bars, of three independent experiments. Mean values were significantly different from the response in solvent-treated cells: *$P<0·05$; mean values were significantly different from the response in cells treated with OTA or AFB1 alone: †$P<0·05$.

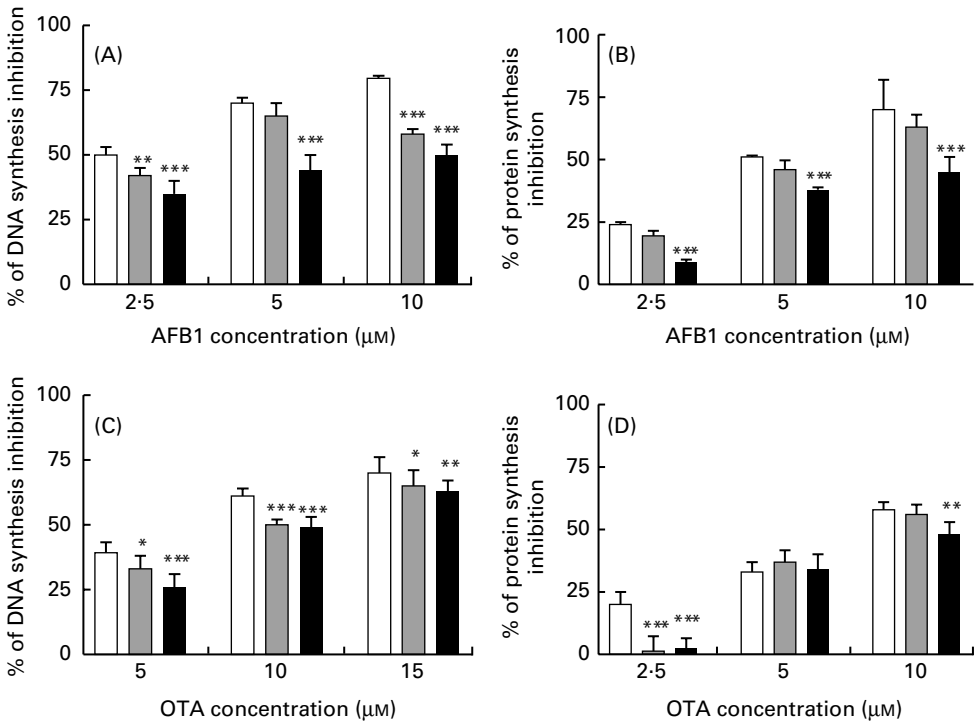

Fig. 5. Influence of 24 h pre-incubation with various concentrations (□, 0 μM (control); ▨, 25 μM; ■, 50 μM) of cyanidin-3-*O*-β-glucopyranoside (C-3-G) on DNA and protein synthesis inhibition induced by aflatoxin B1 (AFB1) and ochratoxin A (OTA) in a human hepatoma-derived cell line (HepG2 cells). Incorporation of (A) [^{3}H]thymidine and (B) [^{3}H]leucine by Hep G2 cells: cultures treated without or with C-3-G were challenged with AFB1 (2·5, 5 or 10 μM) for 24 h and subsequently incubated with [^{3}H]thymidine or [^{3}H]leucine for 3 h at the end of treatment. Incorporation of (C) [^{3}H]thymidine and (D) [^{3}H]leucine by HepG2 cells: cultures treated without or with C-3-G were challenged with OTA (C, 5, 10 or 15 μM; D, 2·5, 5 or 10 μM) for 24 h and subsequently incubated with [^{3}H]thymidine or [^{3}H]leucine for 3 h at the end of treatment. Values are means, with their standard deviation shown as vertical bars, of three independent experiments. Mean values were significantly different from those of the control group (toxin alone): *$P<0·05$, **$P<0·01$, ***$P<0·001$ (one-way ANOVA followed by Newman–Keuls multiple comparison test).

synthesis induced by the treatment with AFB1 and OTA (2·5, 5 and 10 μM; Fig. 6). Moreover, in CaCo-2 cells the treatment with 25 μM-C-3-G had effects qualitatively similar to those of 50 μM-C-3-G treatment.

DNA fragmentation detection

PCR ladder assay was used to detect the development of apoptosis in Hep G2 and CaCo-2 cells following OTA and AFB1 exposure, with or without 24 h pretreatment with C-3-G. DNA fragmentation induced by 24 h of incubation with AFB1 2·5, 5 and 10 μM was shown clearly for Hep G2 and CaCo-2 cells (Fig. 7(A and C)). This effect was dose-dependent. Treatment with 50 μM-C-3-G largely prevented the DNA degradation in both cell lines.

Similarly, incubation of the cells with OTA induced a dose-dependent DNA fragmentation that was not evident at the dose of 2·5 μM, but could clearly be observed with OTA 5, 10 and 15 μM. Treatment with 50 μM-C-3-G prevented DNA laddering that occurred upon DNA fragmentation at each of the OTA concentrations in Hep G2 and CaCo-2 cells (Fig. 7(B and D)).

Caspase-3 colorimetric assay

After incubation of cells for 24 h with the tested concentrations of AFB1 and OTA, caspase-3 activity was measured. Both toxins caused an increased enzyme activity (Fig. 8). The prevention of apoptosis by 50 μM-C-3-G was accompanied by an increase in the inhibition of caspase-3 activity. This inhibition was always significant ($P<0·05$ and $P<0·01$) for the higher C-3-G concentration (50 μM) while the lower one (25 μM) lacked significant effects on Hep G2 and CaCo-2 caspase-3 activity.

Antioxidant activity

Mean values of rac were calculated in the linear concentration range of the sample and the standard. The results are reported in Table 1.

The free-radical scavenging action is due to phenolic –OH group(s) contained in the molecule of the antioxidant (Rice-Evans *et al.* 1996).

Discussion

Since previous studies have implicated oxidative pathways in the toxicity of OTA (Omar *et al.* 1990; Baudrimont *et al.* 1994; Monnet-Tschudi *et al.* 1997) and AFB1 (Cavin *et al.* 1998), natural (vitamins, provitamins, carotenoids, chlorophyll and its derivatives, phenolics, Se) and synthetic compounds with antioxidant properties (butylated hydroxyanisole, butylated hydroxytoluene) could potentially be efficacious in protecting against the toxic effects of these mycotoxins (Galvano *et al.* 2001). The present study was conducted to investigate whether C-3-G, a hydroxylated compound with marked anti-oxidative activity, was capable of protecting Hep G2 and CaCo-2 cells against AFB1- and OTA-induced cell damage. This antioxidant was tested to verify the absence of any cytotoxic or genotoxic effect on Hep G2 and CaCo-2 cells at the concentrations used in the study, and no differences with respect to the control were observed.

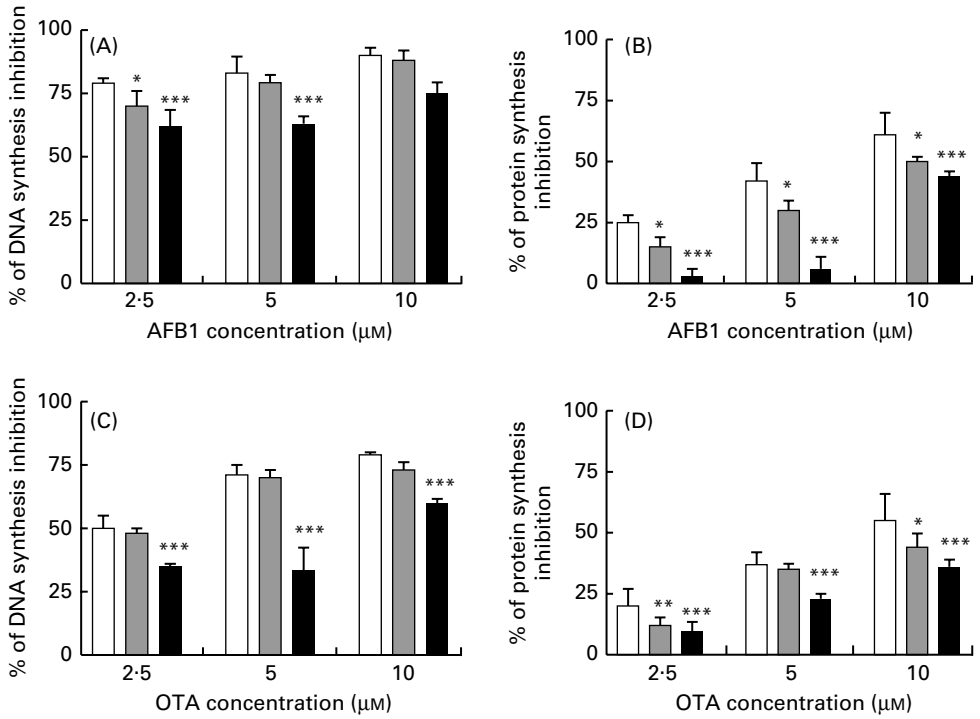

Fig. 6. Influence of 24 h pre-incubation with various concentrations (□, 0 μM (control); ▨, 25 μM; ■, 50 μM) of cyanidin-3-*O*-β-glucopyranoside (C-3-G) on DNA and protein synthesis inhibition induced by aflatoxin B1 (AFB1) and ochratoxin A (OTA) in a human colonic adenocarcinoma cell line (CaCo-2 cells). Incorporation of (A) [^{3}H]thymidine and (B) [^{3}H]leucine by CaCo-2 cells: cultures treated without or with C-3-G were challenged with AFB1 (2·5, 5 or 10 μM) for 24 h and subsequently incubated with [^{3}H]thymidine or [^{3}H]leucine for 3 h at the end of treatment. Incorporation of (C) [^{3}H]thymidine and (D) [^{3}H]leucine by CaCo-2 cells: cultures treated without or with C-3-G were challenged with OTA (2·5, 5 or 10 μM) for 24 h and subsequently incubated with [^{3}H]thymidine or [^{3}H]leucine for 3 h at the end of treatment. Values are means, with their standard deviation shown as vertical bars, of three independent experiments. Mean values were significantly different from those of the control group (toxin alone): $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$ (one-way ANOVA followed by Newman–Keuls multiple comparison test).

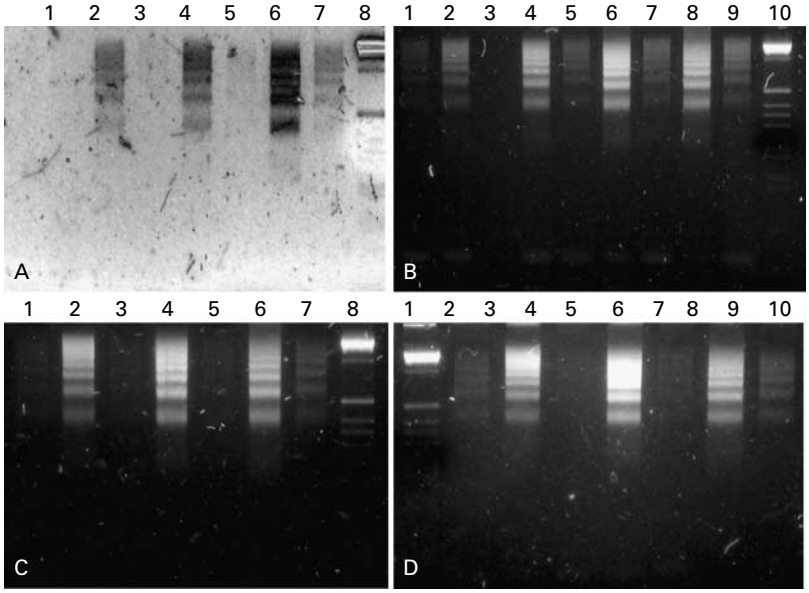

Fig. 7. Apoptosis induced by aflatoxin B1 (AFB1) or ochratoxin A (OTA) and prevention by 24 h pre-incubation with cyanidin-3-*O*-β-glucopyranoside (C-3-G) in a human hepatoma-derived cell line (HepG2 cells) and a human colonic adenocarcinoma cell line (CaCo-2 cells). (A) Hep G2 cells: lane 1, negative control; lanes 2, 4 and 6, cells treated respectively with 2·5, 5 and 10 μM AFB1; lanes 3, 5 and 7, cells pre-treated with 50 μM-C-3-G and then treated respectively with 2·5, 5 and 10 μM AFB1; lane 8, PCR ladder marker. (B) Hep G2 cells: lane 1, negative control; lanes 2, 4, 6 and 8, cells treated respectively with 2·5, 5, 10 and 15 μM OTA; lanes 3, 5, 7 and 9, cells pre-treated with 50 μM-C-3-G and then treated respectively with 2·5, 5, 10 and 15 μM OTA; lane 10, PCR ladder marker. (C) CaCo-2 cells: lane 1, negative control; lanes 2, 4 and 6, cells treated respectively with 2·5, 5 and 10 μM AFB1; lanes 3, 5 and 7, cells pre-treated with 50 μM-C-3-G and then treated respectively with 2·5, 5 and 10 μM AFB; line 8, PCR ladder marker. (D) CaCo-2 cells: lane 1, PCR ladder marker; lane 2, negative control; lanes 3, 5 and 7, cells treated respectively with 2·5, 5 and 10 μM OTA; lanes 4, 6 and 8, cells pre-treated with 50 μM-C-3-G and then treated respectively with 2·5, 5 and 10 μM OTA.

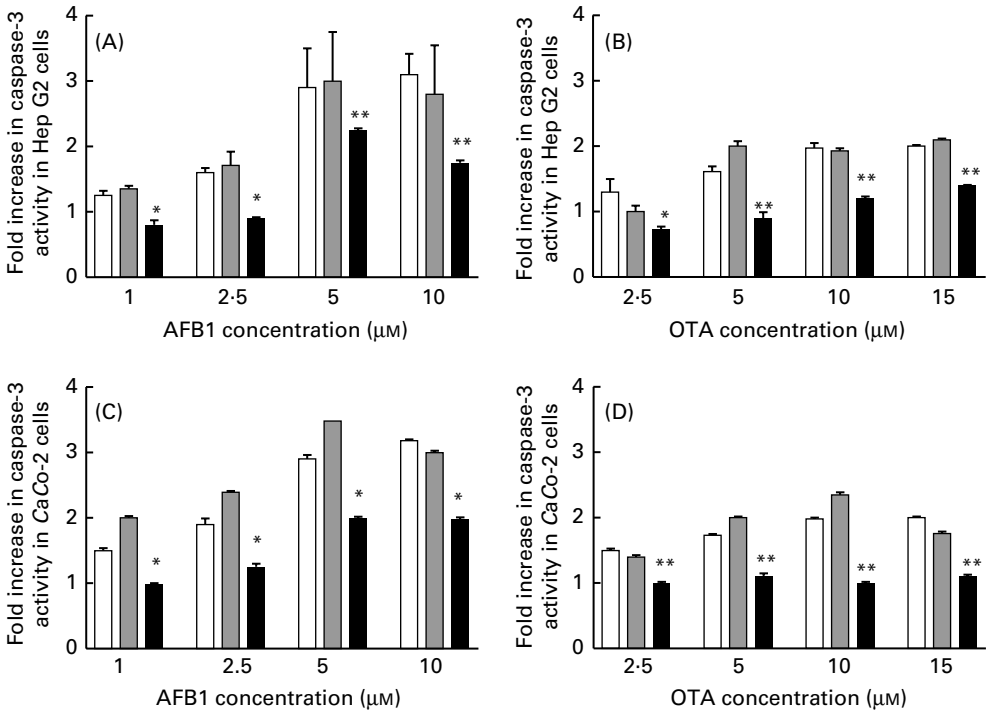

Fig. 8. Influence of 24 h pre-incubation with various concentrations (□, 0 μM (control); ▨, 25 μM; ■, 50 μM) of cyanidin-3-*O*-β-glucopyranoside on activation of caspase-3 induced after 24 h incubation with aflatoxin B1 (AFB1) and ochratoxin A (OTA) in a human hepatoma-derived cell line (Hep G2 cells) and a human colonic adenocarcinoma cell line (CaCo-2 cells). (A) AFB1 (1, 2·5, 5 and 10 μM) in Hep G2 cells (2×10^6); (B) OTA (2·5, 5, 10 and 15 μM) in Hep G2 cells (2×10^6); (C) AFB1 (1, 2·5, 5 and 10 μM) in CaCo-2 cells (2×10^6); (D) OTA (2·5, 5, 10 and 15 μM) in CaCo-2 cells (2×10^6). Quantification was done spectrophotometrically, results are given in relation to untreated cells. Values are means, with their standard deviation shown as vertical bars, of three independent experiments. Mean values were significantly different from those of the control group (toxin alone): $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$ (one-way ANOVA followed by Newman–Keuls multiple comparison test).

Exposure of Hep G2 and CaCo-2 cells to increasing concentrations of both mycotoxins induced cell death in a concentration–time-dependent manner, more evident for AFB1-treated cells. The two toxins also significantly affected total DNA and protein synthesis in a dose-dependent manner, indicating that both cell lines were sensitive to the toxic effects of OTA and AFB1.

In the two cell lines, the effects of the tested concentrations of C-3-G on the OTA- and AFB1-mediated cytotoxicity were similar and differed only quantitatively. Treatment with high C-3-G concentration (50 μM) provided a more efficacious protection compared with the lower concentration (25 μM).

Exposure of Hep G2 and CaCo-2 cells to OTA or AFB1, for 24 h, increased ROS levels in a concentration-dependent manner. The ROS-detecting probe, H_2DCF-DA, detects a wide range of ROS species including $O_2^{\bullet -}$, $HOO^{\bullet}$ and $OH^{\bullet}$. The effects of C-3-G in the present study, which reduced ROS levels significantly, increased after incubation with the two mycotoxins for 24 h, indicating that it could act as a chain-breaking antioxidant. These kinds of antioxidants are particularly effective in scavenging peroxyl and alkoxyl radicals, thus preventing the propagation of lipoperoxidation.

Table 1. Relative activity with respect to concentration (rac) values for the sample studied

Concentration (μm)	Inhibition time (s)	rac	rac_{mean} (SE)
1·33	262	2·33	1·7 (0·2)
1·99	433	1·74	
2·66	584	1·43	
3·32	813	1·29	

Recently, we successfully tested the antioxidant properties of C-3-G from pigmented oranges by a new method based on the inhibitory effects of antioxidants on oscillations of the hydrogen peroxide–acidic iodate–malonic acid–Mn(II)-catalysed system (also known as the BR reaction) which works near the pH of gastric fluids, approximately 2 (Cervellati *et al.* 2001). We found that the antioxidant activity of C-3-G is mainly due to its redox properties, which play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, and decomposing peroxides. The calculated antioxidant activity of C-3-G (rac_{mean} 1·7 (SE 0·2)) is lower than that calculated for rosmarinic acid (rac_{mean} 3·99 (SE 0·08)) in a previous study (Höner & Cervellati, 2002). Consistently, this difference was also observed with respect to the *in vitro* protective properties. Indeed, in previous work (Renzulli *et al.* 2004), C-3-G showed lower protection than rosmarinic acid against the damage induced by mycotoxins.

Pretreatment with C-3-G for 24 h significantly prevented the inhibition of total DNA and protein synthesis induced by OTA and AFB1 in both Hep G2 and CaCo-2 cells. AFB1 induced a clear dose-dependent DNA laddering, also at the lowest concentration. Genomic DNA fragmentation, shown as DNA ladders on gels, a hallmark of apoptosis, was also observed after 24 h incubation with OTA, although visible at higher concentrations.

With the aim of directly measuring the rates of apoptosis induced by AFB1 and OTA, we next tested caspase-3 activity. Even if at high concentration C-3-G alone can induce apoptosis in some human

leukaemia cell lines (Fimognari *et al.* 2004), the anti-apoptotic effect of 50 μM-C-3-G in mycotoxin-challenged Hep G2 and CaCo-2 cells was clearly demonstrated by the almost complete prevention of DNA fragmentation induced by both toxins at all tested concentrations. Besides, the prevention of apoptosis was accompanied by the inhibition of caspase-3 activity, demonstrating that C-3-G is able to inhibit multiple aspects of OTA- and AFB1-induced toxicity in hepatoma cells and colonic adenocarcinoma cells. Interestingly, this significant protective effect can be observed towards the toxicity of both mycotoxins (Renzulli *et al.* 2004). Preventing mycotoxin-induced apoptosis could be also disadvantageous, where exposure induces persistent mutations in the DNA; to clarify this doubt several studies are in progress to evaluate the presence of mutation in surviving cells.

Several studies have proved the benefits of numerous compounds in the diet against the toxicity of a single mycotoxin (Rizzo *et al.* 1994), but this is the second time that a dietary antioxidant has proved to be efficacious on both OTA and AFB1. As co-occurrence of the two toxins in foods and feeds is frequent, an antioxidant able to reduce the effects of both of them could be of great interest. Exposure levels of AFB1 and OTA, as revealed in the plasma of people who have come in contact with these mycotoxins, are still 50–100-fold lower than the concentrations required to cause measurable effects in Hep G2 and CaCo-2 cells, but several drug-metabolising enzymes are less active in the two cell lines than in primary human cells (Knasmüller *et al.* 1998). Therefore, fresh cells would be more sensitive towards OTA and AFB1 than the corresponding cancer cell lines and it needs to be determined in further studies whether these effects also take place in human liver and human colon cells under realistic exposure concentrations.

Although further studies are obviously needed, C-3-G can be considered a potential dietary compound able to counteract the oxidative damages induced by AFB1 and OTA. With this aim it is also important to establish the bioavailability of C-3-G. It was for a long time believed that anthocyanidins were poorly absorbed. This conviction has now been changed by the results of studies showing *in vivo* absorption of C-3-G and other cyanidin-glycosides (Galvano *et al.* 2004). Recent studies have shown that significant amounts of anthocyanins are quickly absorbed from both stomach (Passamonti *et al.* 2003; Talavéra *et al.* 2003) and small intestine (Talavéra *et al.* 2004). In particular the stomach could be a preferential site for anthocyanin absorption (Passamonti *et al.* 2003, Talavéra *et al.* 2003), which in turn could explain the rapid appearance of these compounds in the blood. In any case, tissue distribution of the anthocyanins and their possible transformation after absorption still remain open questions.

The inhibitory action of C-3-G on AFB1- and OTA-induced toxicity is likely to be attributed to its antioxidant power. However, the role that antioxidants, and particularly some polyphenols, could have by producing a coordinated, detoxifying modulation of multiple enzymes of the carcinogens should not be under-evaluated (Aboobaker *et al.* 1997). Indeed, with regard to the mechanism of AFB1 and OTA toxicity, although it is sufficiently demonstrated that their oxidative properties certainly contribute to their cytotoxicity and genotoxicity, information is needed to clarify whether cellular enzyme systems are involved in the toxic pathways of the two mycotoxins (Renzulli *et al.* 2004). New studies are in progress in our laboratory with the aim of establishing whether C-3-G acts mainly as a direct antioxidant or whether its protective properties could be due to the modulation of microsomal enzymes that activate the carcinogen, hence suggesting a potential chemopreventive role against carcinogenesis induced by AFB1.

Acknowledgements

The work was financially supported by grants from the MIUR, PRIN – Cofin 2003, to M. C. G.

References

Abel S & Gelderblom WC (1998) Oxidative damage and fumonisin B1-induced toxicity in primary rat hepatocytes and rat liver in vivo. *Toxicology* **131**, 121–131.

Aboobaker VS, Sarma N, Goswami NU & Bhattacharya RK (1997) Inhibition of microsomal activation of aflatoxin B_1, by 3-dehydroretinol and 3-dehydroretinyl palmitate. *Indian J Exp Biol* **35**, 1125–1127.

Amstad P, Levy A, Emeir H & Cerulti P (1984) Evidence for membrane-medicated chromosomal damage by afflatoxin B1 in human lymphocytes. *Carcinogenesis* **5**, 719–723.

Atroshi F, Parantainen J, Kangasniemi R & Sankari S (1987) Sialic acid, glutathione metabolism, and electrical conductivity in bovine mastitis udder tissue. *J Anim Physiol Anim Nutr* **58**, 200–207.

Baudrimont I, Betbeder AM, Gharbi A, Pfohl-Leszkowicz A, Dirheimer G & Creppy EE (1994) Effect of superoxide dismutase and catalase on the nephrotoxicity induced by subchronical administration of ochratoxin A in rats. *Toxicology* **89**, 101–111.

Belmadani A, Steyn PS, Tramu G, Betbeder AM, Baudrimont I & Creppy EE (1999) Selective toxicity of ochratoxin A in primary cultures from different brain regions. *Arch Toxicol* **73**, 108–114.

Bennett JW & Keller NP (1997) Mycotoxins and their prevention. In *Fungal Biotechnology*, pp. 265–273 [T Anke, editor]. Weinheim: International Thompson Publishing Company.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Briggs T & Rauscher W (1973) An oscillating iodine clock. *J Chem Educ* **50**, 496.

Cavin C, Holzhäser D, Constable A, Huggett AC & Schilter B (1998) The coffee-specific diterpenes cafestol and kahweol protect against aflatoxin B_1-induced genotoxicity through a dual mechanism. *Carcinogenesis* **19**, 1369–1375.

Cervellati R, Höner K, Furrow SD, Neddens C & Costa S (2001) The Briggs–Rauscher reaction as a test to measure the activity of antioxidants. *Helv Chim Acta* **84**, 3533–3547.

Cervellati R, Höner K, Furrow SD, Mazzanti F & Costa S (2004*a*) An experimental mechanistic investigation of the complexities arising during inhibition of the Briggs–Rauscher reaction by antioxidants. *Helv Chim Acta* **87**, 133–155.

Cervellati R, Innocenti G, Dall'Acqua S, Costa S & Sartini E (2004*b*) Polyphenols from *Polygala* spp. and their antioxidant activity. *Chem Biodivers* **1**, 415–425.

Cervellati R, Speroni E, Govoni P, Guerra MC, Costa S, Arnold UW & Stuppner H (2004*c*) *Wulfenia carinthiaca* Jacq., antioxidant and pharmacological activities. *Z Naturforschung* **59**, 255–262.

Cochereau C, Sanchez D & Creppy EE (1997) Tyrosine prevents capsaicin-induced protein synthesis inhibition in cultured cells. *Toxicology* **117**, 133–139.

Fimognari C, Berti F, Nüsse M, Cantelli-Forti G & Hrelia P (2004) Induction of apoptosis in two human leukemia cell lines as well as differentiation in human promyelocytic cells by cyanidin-3-*O*-β-glucopyranoside. *Biochem Pharmacol* **67**, 2047–2056.

Galvano F, La Fauci L, Lazzarino G, Fogliano V, Ritieni A, Ciappellano S, Battistini NC, Tavazzi B & Galvano G (2004) Cyanidins: metabolism and biological properties. *J Nutr Biochem* **15**, 2–11.

Galvano F, Piva A, Ritieni A & Galvano G (2001) Dietary strategies to counteract the effects of mycotoxins: a review. *J Food Prot* **64**, 120–131.

Gautier JC, Holzhaeuser D, Markovic J, Gremaud E, Schilter B & Turesky RJ (2001) Oxidative damage and stress response from ochratoxin A exposure in rats. *Free Radic Biol Med* **30**, 1089–1098.

Gramenitsky EM (1963) *Supravital Staining of Cells and Tissues*, pp. 151. Leningrad: Medgiz.

Höner K & Cervellati R (2002) Measurements of the antioxidant capacity of fruits and vegetables using the BR reaction method. *Eur Food Res Technol* **215**, 437–442.

Höner K, Cervellati R & Neddens C (2002) Measurements of the *in vitro* antioxidant activity of German white wines using a novel method. *Eur Food Res Technol* **214**, 356–360.

International Agency for Research on Cancer (1993) *Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. IARC Monographs on the Evaluation of Carcinogenic Risk to Humans*, vol. 56, Lyon: IARC.

Knasmüller S, Parzefall W, Sanyal R, *et al.* (1998) Use of metabolic competent human hepatoma cells for the detection of mutagens and antimutagens. *Mutat Res* **402**, 185–202.

Kuiper-Goodman T & Scott PM (1989) Risk assessment of the mycotoxin ochratoxin A. *Biomed Environ Sci* **2**, 179–248.

Massey TE, Stewart RK, Daniels JM & Liu L (1995) Biochemical and molecular aspects of mammalian susceptibility to aflatoxin B_1 carcinogenicity. *Proc Soc Exp Biol Med* **208**, 213–227.

Monnet-Tschudi F, Sorg O, Honegger P, Zurich MG, Huggett AC & Schilter B (1997) Effects of the naturally occurring food mycotoxin ochratoxin A on brain cells in culture. *Neurotoxicology* **18**, 831–839.

Omar RF & Rahimtula AD (1983) Possible role of an iron–oxygen complex in 4(S)-4-hydroxyochratoxin A formation by rat liver microsomes. *Biochem Pharmacol* **46**, 2073–2081.

Omar RF, Hasinoff BB, Mejilla F & Rahimtula AD (1990) Mechanism of ochratoxin A stimulated lipid peroxidation. *Biochem Pharmacol* **40**, 1183–1191.

Passamonti S, Vrhovsek U, Vanzo A & Mattivi F (2003) The stomach as a site for anthocyanins absorption from food. *FEBS Lett* **544**, 210–213.

Pietta PG (2000) Flavonoids as antioxidants. *J Nat Prod* **63**, 1035–1042.

Rahimtula AD, Bereziat JC, Bussacchini-Griot V & Bartsch H (1988) Lipid peroxidation as a possible cause of ochratoxin A toxicity. *Biochem Pharmacol* **37**, 4469–4477.

Renzulli C, Galvano F, Pierdomenico L, Speroni E & Guerra MC (2004) Effects of rosmarinic acid against aflatoxin B1 and ochratoxin A-induced cell damage in a human hepatoma cell line (Hep G2). *J Appl Toxicol* **24**, 289–296.

Rice-Evans CA, Miller NJ & Paganga G (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* **20**, 933–956.

Rizzo AF, Atroshi F, Ahotupa M, Sankari S & Elovaara E (1994) Protective effect of antioxidants against free radical-mediated lipid peroxidation induced by DON or T-2 toxin. *J Vet Med Assoc* **41**, 81–90.

Speroni E, Cervellati R, Govoni P, Guizzardi S, Renzulli C & Guerra MC (2003) Efficacy of different *Cynara scolymus* preparations on liver complaints. *J Ethnopharmacol* **86**, 203–211.

Steyn PS & Stander MA (1999) Mycotoxins as causal factors of diseases in humans. *J Toxicol Toxin Rev* **18**, 229–243.

Talavéra S, Felgines C, Texier O, Besson C, Lamaison JL & Rémésy C (2003) Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *J Nutr* **133**, 4178–4182.

Talavéra S, Felgines C, Texier O, Besson C, Manach C, Lamaison JL & Remesy C (2004) Anthocyanins are efficiently absorbed from the small intestine in rats. *J Nutr* **134**, 2275–2279.

Vinson JA, Dabbagh YA, Serry MM & Jang J (1995) Plant flavonoids, especially tea flavonols, are powerful antioxidant using an in vitro oxidation model for heart disease. *J Agric Food Chem* **43**, 5677–5684.

Wang SY & Stretch AW (2001) Antioxidant capacity in cranberry is influenced by cultivar and storage temperature. *J Agric Food Chem* **49**, 969–974.

Yin JJ, Smith MJ, Eppley RM, Page SW & Sphon JA (1998) Effects of fumonisin B_1 on lipid peroxidation in membranes. *Biochim Biophys Acta* **1371**, 134–142.