Abstract. Cancer chemoprevention is considered to be a promising approach for cancer control, as it has been identified by both epidemiological and molecular studies that environmental factors are the major causes of cancer. Chemoprevention can be defined as the use of agents to prevent, inhibit or reverse the process of carcinogenesis. Several epidemiological studies have shown that fruits, vegetables and common beverages, as well as herbs and plants, are rich sources of chemopreventive compounds. In the present report, a battery of in vitro methods for the identification of chemopreventive agents are presented. These methods include: i) inhibition of bleomycin-induced mutations in Salmonella typhimurium TA102 cells, ii) inhibition of bleomycin-induced sister chromatid exchanges (SCEs) in human peripheral blood lymphocytes, iii) protection from mitomycin C-induced DNA strand breakage and iv) inhibition of topoisomerase I DNA relaxation. The first three methods are also used for the identification of agents which prevent reactive oxygen species (ROS)-mediated DNA damage.

Cancer is one of the major causes of death in the world, despite improvements in its early detection and treatment (1). According to the multistage evolution of cancer, carcinogenesis can be considered as a process that involves abnormal metabolic changes by which a normal cell is transformed to a cancerous one (2). Thus, it is believed that, in general, carcinogenesis includes three stages: initiation, promotion and progression, which are caused by different mechanisms (3-5). The initiation stage is an irreversible event that occurs when a normal cell is exposed to a carcinogen that causes DNA damage in genes controlling proliferation, differentiation and apoptosis. The previous mutations result in a cell (initiated cell) that has the ability to perform, under the appropriate conditions, a greater number of mitotic divisions than a normal cell (6). During the second stage of carcinogenesis, promotion, the initiated cell, under the effect of mitogenic agents, produces a clone of mutated cells. In one of these cells, a crucial mutation occurs which transforms it to the first pre-cancerous cell. The promotion stage is reversible and usually needs several years to become established (4, 7). At the third stage, progression, the pre-cancerous cell is converted to a cancerous one due to the accumulation of genetic changes which yield the cancerous phenotype, namely a cell clone with increased proliferative capacity, invasiveness and metastatic potential (5). Like the initiation stage, the progression stage is irreversible.

Given that 80% of human cancer is caused by environmental agents, as indicated by epidemiological studies (8), cancer prevention is being recognized as an important strategy for cancer control (9). Cancer prevention includes reduction of human exposure to environmental carcinogens, changes that favor a healthier lifestyle and chemoprevention. Chemoprevention is considered to be a promising approach to reduce the incidence of human cancer (10) and can be defined as the prevention, inhibition or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or naturally occurring constituents of the diet (11). Specifically, fruits, vegetables and common beverages, as well as herbs and plants, have been shown to be rich sources of various chemical compounds with potential chemopreventive activity (12-15).

In the present report, a battery of cell- and enzyme-based in vitro tests are presented. These short-term, inexpensive and simple tests can be used for both the screening of potential chemopreventive agents and the mechanisms underlying their action, particularly, for the...
detection of agents which protect from DNA damage caused by reactive oxygen species (ROS). The term ROS is a collective term that includes oxygen-centered radicals such as superoxide anion (O$_2^•^•$) and hydroxyl radical (OH•), as well as some non-radical derivatives of oxygen such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (1O$_2$) (16). ROS are constantly produced in cells by cellular metabolism and by exogenous agents. They are essential for life because they are involved in cell signaling and are used by phagocytes for their bactericidal action (16). However, these species can react with various biomolecules in cells, such as lipids, proteins and DNA, and can cause damage to these molecules (17, 18). Thus, an imbalance between the production of ROS and antioxidant defenses leads to oxidative stress, which might be linked to degenerative processes such as cancer, aging, cardiovascular and neurodegenerative diseases (19-21). In particular, ROS-mediated DNA damage is associated with increased risk of developing cancer (23). Therefore, agents that reduce oxidative DNA damage should decrease the risk of cancer development (24).

The first of the assays presented is the mitomycin C (MMC)-induced DNA breakage. MMC, a quinone isolated from Streptomyces caespitosus, is used in combination with other agents for the treatment of a large number of advanced human cancers. Upon reduction, MMC is transformed into a semiquinone radical which reacts with oxygen, resulting in the production of hydrogen peroxide (H$_2$O$_2$) (25). In the presence of Fe$^{+2}$, the OH• is produced from H$_2$O$_2$ through the metal-catalyzed Fenton reaction (reaction 1).

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH• + OH^- \quad (1)$$

As mentioned above, OH• are strong oxidants which cause DNA strand breaks (26). A single-strand break in supercoiled double-stranded circular DNA results in the formation of open circular DNA, and double-strand breaks result in the formation of linear DNA (27). In agarose gel electrophoresis, the three DNA forms are separated because supercoiled DNA migrates faster than open circular DNA (which is less compact) and linear DNA migrates in an intermediate position between supercoiled and open circular DNA. An antioxidant agent, which prevents DNA strand breaks either by scavenging the OH• or by chelating the Fe$^{+2}$ and, thus, blocking the Fenton reaction, would inhibit the conversion of supercoiled DNA to the open circular and linear forms (28, 29).

The second method presented is a short-term bacterial antimutagenic assay based on the Ames Salmonella typhimurium mutagenicity test. Ames and co-workers (30, 31) developed a number of Salmonella typhimurium strains in order to detect a wide range of chemical substances that cause gene mutations. These strains are histidine-dependent and carry mutations in various genes in the histidine operon. When these cells are grown on a minimal media agar plate containing traces of histidine, only those cells that revert to histidine independence are able to form colonies. The number of spontaneously revertant colonies per plate is relatively constant for each strain. However, when a mutagen is added to the plate, the number of revertant colonies is increased in comparison with the plates that do not contain the mutagen (32). Apart from the detection of mutagen compounds, the Ames Salmonella assay can be used for the detection of antimutagens (33-35). In this case, the number of revertant colonies growing on the plates containing both the mutagen and the antimutagen is decreased in comparison with the plates containing only the mutagen. For the detection of antimutagens acting through antioxidant mechanisms, the TA102 strain is usually used, because it is sensitive to oxidant mutagens (30, 32, 36, 37).

In the present study, bleomycin A$_2$ (BLM) was used as an oxidant agent. BLM is the collective name given to a family of glycopeptide antibiotics which are produced by Streptomyces verticillus and are used for the treatment of several human cancers. It is thought that the mutagenicity of BLM involves binding to DNA and activation of molecular oxygen in the presence of Fe$^{+2}$ to produce ROS such as O$_2•^-•$ and OH•, which induce single- and double-strand DNA breaks and degradation of the deoxyribose sugar (38, 39). In addition, it would be informative if more than one oxidant were used (e.g. BLM, H$_2$O$_2$ and tert-butyl hydroperoxide) in TA102 cells for the screening of antioxidant substances, because the different oxidants act by different mechanisms (40, 41).

In contrast to the Ames test, in which prokaryotic cells are used, there are also other antimutagenic assays in which eukaryotic cells are used. A cytogenetic biomarker, which is applied for the assessment of chemopreventive agents in peripheral blood lymphocytes, is the sister chromatid exchanges (SCEs) (42-44). A SCE represents the exchange of homologous chromosomal segments between two sister chromatids and involves the breakage of four strands of DNA (two double helices) and reunion of these strands in their new location (45). Although SCE has been considered to be a very sensitive method for the detection of chemical mutagens and carcinogens since the 70s (46, 47), the molecular basis of these chromosomal interchanges is still obscure (48). A number of studies have reported that ROS increase the frequency of SCEs (49), while antioxidants and micronutrients suppress the former increase (42, 50). As in the Ames assay, BLM was used as an oxidant agent for the induction of SCEs in the present study.
Another approach to detect chemopreventive substances is to test their ability to inhibit enzymes showing elevated activity in cancerous cells such as topoisomerase I (51, 52) and II (53), telomerase (54), ornithine decarboxylase (55) and DNA polymerase (56). In this report, the topoisomerase I (topo I) relaxation assay was applied. DNA topoisomerases are enzymes that catalyze the interconversion of topological isomers of DNA molecules. Specifically, DNA topo I acts by the sequential breakage and reunion of one strand of DNA and has an important role in replication, recombination, transcription, chromosome condensation and maintenance of genome stability (57). Because it is involved in vital cellular processes, it is considered to be a good target for anticancer agents (58).

Materials and Methods

Chemicals, culture reagents, DNA and enzymes. MMC and BLM were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan) and Nippon Kayaku Co., Ltd. (Tokyo, Japan), respectively. pBR322 was from New England BioLabs Ltd. (Hertfordshire, UK). Human topo I was obtained from TopoGen Inc., (Ohio, USA). Ethylenediamine tetraacetic acid was purchased from Panreac (Barcelona, Spain). Nutrient broth no. 2 was from Oxoid (Basingstoke, UK). RPMI 1640, L-glutamine, penicillin, streptomycin, phytohemaglutinin and fetal calf serum were from Biochrom (Berlin, Germany). Ferrous sulfate and Giemsa were purchased from Merck (Darmstadt, Germany). Caffeic acid, ferulic acid, protocatechuc acid, rutin hydrate, gallic acid, 5-bromo-2-deoxyuridine and all other chemicals were obtained from Sigma-Aldrich (St. Louis, USA).

MMC-induced DNA strand cleavage. This test was used to test the antimutagenic activity of a methanicole white grape extract from a Greek Vitis vinifera variety (Assyrtiko Santorini). DNA strand breakage was measured by the conversion of supercoiled pGem-3z double-stranded DNA to open circular and linear forms. The DNA strand cleavage test was performed by the procedure of Yamagishi et al. (29). pGem-3z (37.5 ng/10 µl) was treated with 143 µM FeSO 4.7H 2O, 78 µM EDTA and 0.004% MMC, in the presence or absence of the test sample, and the mixture was incubated at 37°C for 60 min. The volume of the reaction was 10 µl. Following incubation, the samples were mixed with 3 µl of gel loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and separated by electrophoresis in a horizontal 0.9% agarose gel with 40 mM Tris-acetate and 2 mM EDTA pH 8.0 as the running buffer, at 5 V/cm for 90 min. After gel electrophoresis, the DNA bands were stained with ethidium bromide at 0.5 µg/ml for 30 min, followed by destaining in water for 30 min. The gels were photographed by UV transillumination using a Polaroid DS-34 camera and the image was quantified using Gel-Pro Analyzer version 3.0 (MediaCybernetics, Silver Spring, USA) software. It should be noted that the commercially available pGem-3z DNA contained approximately 10% open circular DNA prior to treatment. The percent inhibition of MMC-induced DNA cleavage was calculated by the following formula:

\[ \frac{(S-S_0)/(S_{control} - S_0)}{1} \times 100 \]

Where S control is the percent of supercoiled DNA in the control lane (without MMC and test compounds), S 0 is the percent of supercoiled DNA in the lane without test compounds but with MMC, and S is the percent of supercoiled DNA in the lane with test compounds and MMC. Three independent experiments were carried out for each compound. The extract dissolved in methanol resulted in a final methanol concentration of 0.03%. At this concentration, methanol had no effect on MMC activity.

Salmonella tester strain. The bacterial strain Salmonella typhimurium TA102 was checked routinely to confirm genetic features, using the procedure outlined by Maron and Ames (59). For each experiment, a frozen stock culture was allowed to thaw at room temperature. Three hundred µl of the stock culture were then used to inoculate 30 ml of autoclaved Oxoid nutrient broth no. 2. Ten hours prior to the experiment, the inoculated cultures were placed on a shaker (100 rpm) and incubated in the dark at 37°C until the cells reach a density of 1 to 2X10 9 colony-forming units (CFU/ml, O.D.540 between 0.1 and 0.2).

The Ames Salmonella mutagenicity test. The standard plate incorporation procedure (59, 32) was used for the antimutagenicity testing of an aqueous white grape extract from a Greek Vitis vinifera variety (Assyrtiko Santorini) against BLM-induced mutagenicity. The extraction and isolation procedure were as previously described (28). The extract was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.65%, at which it had no effect on BLM mutagenicity. The final concentration of BLM was 0.5 µg/plate, because this concentration was found to be the most suitable by a dose-response curve (37). All the antimutagenicity mutation assays were carried out in triplicate and on two separate occasions. In each experiment, the following substances were added in screwed sterile tubes maintained at 45°C: 2 ml top agar; 50 µl bleomycin solution; 50 µl plant extract solution; 100 µl bacterial culture. The contents of the tubes were mixed and poured onto the surface of glucose minimal agar plates. When the top agar was hardened, the plates were inverted and placed in a 37°C incubator for 48 h. Afterwards, the His + revertant colonies were counted. Before counting, the number of revertant colonies were microscopically checked for thinning or absence of the background lawn and/or presence of microcolonies, which are considered as indicators of toxicity (32) induced by a test chemical. Each assay included both positive (bleomycin alone) and negative (no mutagen) controls. Also, each antioxidant was monitored for the possible induction of mutations.

The number of induced revertants was obtained by subtracting the number of spontaneous revertants from the number of revertants on the plates containing the mutagen and/or antimutagen. For statistical analysis, the Student’s t-test was applied to calculate the confidence intervals (p<0.05). Inhibition of (%) mutagenicity was calculated as follows:

\[ \frac{\text{revertants / plate with BLM} + \text{test compound} - \text{(spontaneous revertants)}}{\text{revertants / plate with BLM alone} - \text{(spontaneous revertants)}} \times 100 \]

Sister chromatid exchange (SCE) test. This assay was used to identify the potential chemopreventive activity of five plant polyphenols.
(caffeic acid, ferulic acid, gallic acid and protocatechuic acid) against BLM-induced clastogenicity in human blood lymphocytes. Heparinized blood was obtained by venipuncture of an arm from healthy blood donors (20 and 35 years old). They did not smoke and were not taking medicine. Whole blood cultures were initiated by adding 10 drops of blood to each culture flask containing 5 ml RPMI 1640 supplemented with 15% fetal calf serum, 4mM L-glutamine, 100 U/ml penicillin, 100 Ìg/ml streptomycin and 0.1 ml phytohemaglutinin (Biochrom). 5-bromo-2’-deoxyuridine at a final concentration of 10 Ìg/ml, 100 ìl of BLM solution at a final concentration of 2.5 Ìg/ml and 100 ìl of tested compound solutions at a final concentration of 20 ̪ were added to the cultures at the beginning of the 72-h incubation period, in the dark at 37ÆC. The assay included both positive (BLM alone) and negative (without BLM or test compounds) cultures. The metaphases were blocked during the last 2 h of the incubation period with colchicine at a final concentration of 0.5 Ìg/ml. After the harvesting, hypotonic treatment was conducted with prewarmed 0.075 M potassium chloride (KCl) solution following a 20-min incubation at 37°C. After centrifugation at 1500 rpm for 10 min, the cells were fixed three times with a fixative consisting of three parts methanol and one part glacial acetic acid, then the cells were stored at ~20°C overnight. Slides were prepared and stained by the fluorescence plus Giemsa (FPG) method (60) with slight modifications. The slides were coded and scored for SCEs by one investigator to minimize observer bias. The frequency of SCEs at each culture was determined randomly in 30 second division metaphases with ≥42 chromosomes and presented as SCEs per cell. For statistical analysis, the Student’s t-test was applied (p<0.05).

Results and Discussion

MMC-induced DNA breakage. This assay was used to evaluate the potential chemopreventive activity of a methanolic extract from the Greek Vitis vinifera variety (Assyrtiko Santorini) against ROS-mediated DNA breakage. The extract inhibited MMC-induced DNA cleavage by 42 and 33% at a concentration of 350 and 150 ìg/ml, respectively (Figure 1).
This inhibitory activity suggests that the protective activity of grape extracts against ROS-mediated DNA strand cleavage may be another mechanism by which the grape extracts exert their chemopreventive and anticarcinogenic activities.

**The Ames/Salmonella mutagenicity test.** This test was used to test the antimutagenic activity of an aqueous extract from the Greek *Vitis vinifera* variety (*Assyrtiko Santorini*), against BLM-induced mutagenicity. The number of revertant cells was reduced by 17% and 35% at concentrations of 3 mg/plate and 6 mg/plate, respectively (Figure 2). The antimutagenic effect of this extract may be attributed to the polyphenols present in it, as it is known that the plant polyphenols, especially those found in grapes, have antioxidant (61) as well as metal chelating (62) properties which could inhibit the DNA damage activity of BLM. As mentioned above, BLM activates oxygen in the presence of Fe$^{+2}$, leading to the production of ROS which cause single- and double-strand breaks.

**The SCEs test.** This assay was used to assess the chemopreventive effect of plant polyphenols (caffeic acid, ferulic acid, gallic acid and protocatechuic acid) on BLM-induced clastogenicity in human peripheral blood lymphocytes. Caffeic acid and rutin hydrate inhibited the clastogenic activity of BLM at a concentration of 20 μM by 65% and 57%, respectively (Table 1), while the other polyphenolics had no significant effect on BLM activity. The chelating properties of caffeic acid could account for its inhibitory activity against BLM-induced DNA damage. It is noteworthy that ferulic acid did not inhibit BLM activity, although it has a similar chemical structure to caffeic acid. The latter has a hydroxyl group substituted for a methoxyl group. It has been proposed that the *ortho*-hydroxyl structure is important for the metal chelation properties of caffeic acid (40, 63). Furthermore, caffeic acid has been found to inhibit BLM-induced mutagenicity in *Salmonella typhimurium* TA102 cells (37).

**Topoisomerase I relaxation assay.** This assay was used to assess the inhibitory activity of an aqueous extract of the Greek *Vitis vinifera* variety, *Assyrtiko Santorini*, against human topo I. The extract inhibited relaxation of pBR322 supercoiled DNA by 88% and 97% at 400 μg/ml and 800 μg/ml, respectively (Figure 3). The same extract has also been shown to inhibit

![Figure 3. Inhibition of Assyrtiko variety aqueous grape extract on the catalytic activity of human topo I. (A) Lane 1, pBR322 DNA; lane 2, DNA with 1 unit of human topo I; lanes 3-6, 50, 200, 400 and 800 μg/ml of extract. Positions of supercoiled (SC) and relaxed (R) DNA are indicated. (B) Percent inhibition. The experimental method is described in the Materials and Methods section. Results are presented as mean ± SD of three independent experiments.](image-url)
topo I from wheat germ (28). Several plant polyphenols have been found to inhibit topo I. The inhibition of topo I by grape extract may be one of the mechanisms by which grape extracts inhibit the growth of human cancer cells (64). As some of the polyphenolics present in grapes have been reported to inhibit topo I (65, 66), they may account for the inhibitory activity of the grape extract against topo I.

References

Stagos et al: Antioxidant / Anticarcinogenic Activity of Plant Extracts


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