Influence of the antioxidant N-acetylcysteine and its metabolites on damage induced by bleomycin in PM2 bacteriophage DNA

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Bleomycin is considered to be a useful model compound for studying environmental carcinogenesis, due to its broad spectrum of DNA damaging properties. In addition, bleomycin is a useful antitumor drug because of its cytotoxic properties. To investigate the influence of the antioxidant N-acetylcysteine and its metabolites glutathione and cysteine on bleomycin-induced DNA damage and more importantly to gain insight into the biological relevance of such damage, PM2 DNA was exposed to Cu2+-bleomycin in the presence and absence of the thiols N-acetylcysteine, glutathione and cysteine. It was found that the presence of these thiols led to a considerable enhancement of bleomycin-induced single- and double-strand breaks and a concomitant decrease in the biological activity of PM2 DNA in a dose-dependent way. A similar observation was made when ascorbic acid was used. Bleomycin showed no DNA damaging activity when PM2 DNA was pretreated with the strong Fe ion chelator desferal and its activity was strongly inhibited by the addition of Cu2+ ions or under hypoxic (N2) conditions. Cu2+-bleomycin under our conditions is not active by itself, but most probably after binding to DNA exchanges Cu2+ for Fe3+ bound to DNA. Fe3+-bleomycin is then reduced to Fe2+-bleomycin, a process potentiated by the added antioxidants, and subsequently activated by O2. The contribution to biological inactivation of bleomycin alone or in the presence of ascorbic acid is only ~15%. The contribution to lethality in the presence of thiols is higher. These results indicate that ascorbic acid only enhances the DNA damaging properties of bleomycin, whereas the thiol compounds in addition influence the type of DNA damage. The remainder of the biological inactivation is probably caused by double damage, such as single-strand breaks with closely opposed alkali-labile sites or base damage.

Introduction

A new goal in cancer research is to protect persons against the introduction of DNA damage in order to prevent mutational events that may induce carcinogenesis. To investigate this, population-based studies are currently conducted to delay or inhibit the process of carcinogenesis through supplementation with protective agents (1). These so-called chemoprevention strategies are not feasible for the whole population. However, for selected groups of people with an increased risk of developing cancer chemoprevention has better prospects. One such specific group that could possibly benefit from chemoprevention are curatively treated head and neck squamous cell carcinoma (HNSCC*) patients who are at high risk of development of second primary tumors. For this latter group chemoprevention trials have been performed by supplementation with free radical scavengers (2) with the aim of protecting against free radical-mediated DNA damage. An important and presently used chemopreventive drug is the thiol N-acetylcysteine (NAC), a potent antioxidant, which is often used as a mucolytic drug in lung diseases and which has only minor toxic side effects (3). The selection of NAC for clinical trials was based on its anti-mutagenic (4) and anti-carcinogenic (5) activity in bacteria and murine model systems.

It is well established that people differ in their susceptibility to cancer. In an attempt to measure this phenomenon it has been found that peripheral blood lymphocytes of HNSCC patients are very sensitive to the clastogenic effects of bleomycin (BLM) (6,7). This finding was based on in vitro measurements of chromosomal aberrations in mitogen-stimulated lymphocytes challenged with BLM. This antibiotic and cytotoxic drug may be considered a very useful test compound, since it has a broad spectrum of DNA damaging properties comparable with carcinogens to which the patients are exposed (8). An important mechanism of the genotoxic effects of BLM is the formation of single-strand breaks (SSB), double-strand breaks (DSB) and apurinic/apyrimidiniec (AP) sites containing oxidized deoxyribose moieties by the activated BLM and, possibly in part, by hydroxyl radicals generated via free radical-mediated reactions (9). Theoretically, antioxidants, such as thiols, may be able to protect against BLM-induced DNA damage through scavenging free radicals. On the other hand, instead of a protective effect, a potentiation of DNA damage by thiols has also been reported (10,11). This latter effect is interesting for the cytotoxic properties of BLM, which may imply an increased efficacy for the treatment of cancer.

The purpose of the study presented in this paper was to investigate whether the thiol NAC can interfere with induction of DNA damage by BLM and whether this interference leads to protection or potentiation. The influence on DNA damage was studied in a cell-free system, in which supercoiled PM2 DNA was exposed to BLM in combination with NAC. It has been reported that NAC is very easily deacetylated even before it enters the cell and is converted into cysteine (Cys) (12). Moreover, a significant increase in glutathione (GSH) concentration has been found in the plasma of rats after oral administration of 100 mg NAC (4) and in humans after a daily intake of 600 mg (13). Since in our cell-free system no enzymes were present to convert NAC, experiments were also

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performed with addition of these two active metabolites to our system. To compare the results obtained with these thiol with another well known potent antioxidant experiments were also performed with ascorbic acid (Asc) (14). The PM2 system not only allows us to determine the kind and number of certain types of DNA lesions, such as SSB and DSB, by agarose gel electrophoresis, but, in addition, the influence of these lesions on the biological activity of DNA could be measured. This latter measurement was performed by transformation of the exposed PM2 bacteriophage DNA into its host bacteria (A. espejiana).

Materials and methods

**Bacteriophage DNA and bacterial strains**

PM2 DNA was obtained from Boehringer Mannheim (Mannheim, Germany). For biological activity measurements the bacterial strain A. espejiana (Pseudomonas BAL-31) was used (National Collection of Industrial and Marine Bacteria, Aberdeen, UK; catalogue no.1879). From this strain we isolated a variant, A. espejiana PM2, which cannot be infected by PM2 bacteriophages (15,16).

**Exposure conditions**

Ten microliters of PM2 DNA (30 ng/μl) were dissolved in 10 μl 250 mM phosphate buffer (KH2PO4/K2HPO4 1:4, pH 7.4) and exposed to 10 μl of several concentrations of Cu–BLM (a gift of Lundbeck, Amsterdam, The Netherlands) and 20 μl H2O (or several concentrations of antioxidant) in a 0.5 ml Eppendorf vial for 1 h at 37°C. The reaction was stopped by adding 10 μl 30 mM EDTA. Stock solutions of antioxidants (1 mM) were prepared in 250 mM phosphate buffer and adjusted to pH 7.4. Asc and Cys were obtained from Sigma (St Louis, MO). GSH was purchased from Boehringer Mannheim (Mannheim, Germany). NAC was obtained from BUFA BV (Utrecht, The Netherlands). All solutions were freshly prepared before performing the incubations.

In a single experiment the iron concentration was reduced by dialyzing the DNA against 0.3 mM desferal (in tridest) for 24 h, followed by a 1 week dialysis against tridest. To determine the influence of copper, BLM activity was also determined at 10 and 50 μM CuCl2.

**Measurements of strand breaks**

Strand breaks were measured using 0.8% agarose gel electrophoresis (Pronarose Hispanic; SphaeroQ, Leiden, The Netherlands) in TAE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 7.6, with acetic acid) containing 0.5 μg/ml ethidium bromide. To 10 μl of the exposed DNA (50 ng) were added 2 μl sample buffer (15% Ficoll, 5 mM EDTA, 0.25% bromophenol blue) and the whole mixture was loaded on the gel. After electrophoresis at 50 V, 100 mA for 2 h the gel was illuminated with UV and the image was stored using a Charge Coupled Device videocamera coupled to an Image Processing system (Cybertech, Berlin, Germany). DNA in the covalently closed circular (CCC), open circular (OC) and linear forms was quantified using two-dimensional densitometry. A correction factor of 1.2 was used to compensate for the reduced binding of ethidium bromide to CCC DNA. The number of SSB (PSSS) or DSB (PDS) was calculated from the exponential decrease in CCC and OC DNA molecules. According to the Poisson statistics the probability that a DNA molecule will be broken is e⁻N. In practice this is denoted as PSSS = -ln(intensity measured in the CCC band×1.2)/intensity of all bands (total DNA)] and PDS = -ln([intensity of the CCC band×1.2] + intensity of the OC band/intensity of all bands (total DNA)]. The number of heat-labile lesions was measured by the decrease in CCC DNA after an additional incubation of 2 h at 45°C. The alkali-labile lesions were measured by addition of 7.2 μl 0.96 M NaOH (final pH 12.5) at 45°C to the 60 μl exposed DNA sample. After 75 min this was neutralized by adding 23 μl 0.96 M NaOH (final pH 12.5) at 45°C to the 60 μl exposed DNA sample. After 75 min this was neutralized by adding 23 μl 0.96 M NaOH (final pH 12.5) at 45°C to the 60 μl exposed DNA sample. After 75 min this was neutralized by adding 23 μl 0.96 M NaOH (final pH 12.5) at 45°C to the 60 μl exposed DNA sample.

**Determination of biological activity of the exposed PM2 DNA**

The bacteria were cultured until an OD of 0.5 was reached (measured at 570 nm) at 25°C in BAL broth medium containing 8 g Nutrient Broth (Oxoid Ltd, Basingstoke, UK) in AMS solution (10 mM KCl, 0.5 M NaCl, 50 mM MgSO4 and 10 mM CaCl2 in MilliQ water). Spheroplasts were prepared by resuspending a pellet of 50 ml A. espejiana PM2 in 1 ml NaCl-sodium citrate (1 M NaCl, 20 mM Tris–HCl, pH 7.0) and 0.3 ml 30% (w/v) bovine serum albumin (Sigma, St Louis, MO). During this whole procedure the solutions were kept on ice. Lysozyme solution (100 μl 2 mg/ml freshly prepared in 10 mM phosphate buffer, pH 7.0) was added and after 5 min 4.9 ml EDTA broth (BAL broth without MgSO4, but with 3 mM Na2EDTA) were incubated for 6 min. Finally, a mixture of 260 μl 1 M MgSO4 and 40 μl 1% (w/v) protease (Sigma; freshly prepared in 10 mM phosphate buffer, pH 7.0) was incubated for 10 min, after which the spheroplasts remained competent on ice for several hours. Transformation was performed by incubating 100 μl spheroplasts with 150 μl PM2 DNA (12 μl sample (60 ng DNA) plus 138 μl 10 mM phosphate buffer, pH 7.0) for 10 min on ice. The mixtures were cultured in 800 μl BAL agar broth for 4 h at 25°C. Biological activity was measured as plaque forming units (p.f.u./ml).

The induction of lethal hits was calculated from the exponential decrease in biological activity using the Poisson statistic: -ln(phosphate). Asc and Cys were considered to be almost 100% lethal, so the contribution of DSB to biological inactivation could also be calculated. To achieve this the mean number of DSB of exposed DNA was divided by the number of lethal hits calculated from the biological activity assay (×100%). As controls we used fresh PM2 DNA and DNA that had undergone the whole procedure except for addition of any damaging compound.

**Results**

The influence of NAC, Cys, GSH and Asc on DNA strand break induction by BLM

Before screening for a possible influence of NAC on BLM-induced DNA damage a dose–response experiment was performed in which PM2 DNA was exposed to increasing concentrations of BLM (Figure 1). Detectable induction of both SSB and DSB occurred above a BLM concentration of 1 μM. From 1 to 50 μM BLM a linear (r = 0.98) dose–response effect was noted. The induction of SSB and DSB appeared to proceed in a parallel fashion in which the DSB:SSB ratio was ~1:8. At ~50 μM a plateau was reached. For further study of possible influences of antioxidants two concentrations of BLM were taken, 2 and 20 μM. At 2 μM potentiation of the induction of breaks can be accurately assessed, whereas at 20 μM BLM a possible protective effect of the antioxidants can be measured. Figure 2 shows the induction of DSB at a fixed concentration of 20 μM BLM with increasing concentrations of the antioxidants. A clear potentiation of DNA damage was observed. For SSB comparable results were obtained and this was also found when the BLM concentration was fixed at 2 μM (data not shown). At higher concentrations, especially,
potentiation by Cys tended to be stronger than by NAC and GSH. The decrease in DSB at high Cys concentrations, shown in Figure 2, is due to considerable loss of material by excessive breakdown of the DNA and therefore \( P_{ds} \) is underestimated. Exposure of the DNA to antioxidants without BLM showed no DSB and only a limited number of SSB (2 mM Cys, \( P_{ss} = 0.5 \)).

To investigate whether this potentiation by thiol compounds is a common feature of antioxidants, Asc was also tested. A broad range of Asc concentrations was used (0.2 \( \mu M \)-10 mM) with 0.2-20 \( \mu M \) BLM. Figure 3 shows a representative example of the influence of Asc on DSB induction by BLM. Asc was very effective in enhancing BLM-induced damage. Virtually no DSB were induced by Asc alone (10 mM Asc, \( P_{ds} = 0.31, P_{ss} = 1:100 \)). Potentiation of BLM-induced DSB after pre-incubation of BLM with Asc for 1 h at 37°C was slightly less (Figure 3).

The presence of iron ions appeared to be essential for BLM activation. When all iron ions were removed from the DNA by pre-treatment with desferal no BLM-induced DNA damage could be detected. A similar observation was made when copper ions were added to the reaction mixture. Under these conditions of high copper concentration BLM activity was inhibited. Besides iron ions, \( O_2 \) is also necessary for BLM activation. When the \( O_2 \) concentration was considerably reduced by replacing air in the tubes and solutions with nitrogen (\( N_2 \)) formation of BLM-induced SSB is strongly and that of DSB completely inhibited, as shown in Figure 4. This was found both in the presence and absence of antioxidants.

In addition to SSB and DSB, heat-labile and alkali-labile sites, i.e. lesions which can be converted into strand breaks by heat or alkali treatment (17) may be expected to be introduced by exposure of DNA to BLM. No heat-labile sites appeared to be induced in PM2 DNA after a 1 h exposure to 2 or 20 \( \mu M \) BLM, since no extra breaks could be detected upon additional incubation of the exposed DNA at 45°C for 2 h. On the other hand, a small number of alkali-labile sites, detected by an additional incubation of the exposed DNA at

![Fig. 2. The influence of NAC, Cys and GSH on BLM-induced strand breakage. PM2 DNA was exposed to 20 \( \mu M \) BLM for 1 h at 37°C in the presence of various concentrations of the thiol compounds. Means of the induced DSB are given from two separate experiments (± SD).](https://academic.oup.com/carcin/article-abstract/17/2/327/269823/)

![Fig. 3. A representative experiment of the induction of PM2 DNA strand breakage by Asc. The PM2 DNA was incubated for 1 h at 37°C with several concentrations of Asc. This was either performed without BLM (control) or with a fixed BLM concentration of 2 or 20 \( \mu M \). The pre-incubation with Asc was performed at 10 \( \mu M \) BLM for 1 h before addition of DNA. Results are expressed as the probability of a DNA molecule containing a DSB (\( P_{ds} \)).](https://academic.oup.com/carcin/article-abstract/17/2/327/269823/)

![Fig. 4. The influence on the induction of SSB in PM2 DNA by BLM and thiol compounds is shown both under normal (aerobic) conditions and under \( N_2 \) (g). No DSB were induced under anaerobic conditions.](https://academic.oup.com/carcin/article-abstract/17/2/327/269823/)

![Fig. 5. Exposed DNA samples (1 h at 37°C to 20 \( \mu M \) BLM with and without 200 \( \mu M \) thiols or 20 \( \mu M \) Asc) were additionally incubated at pH 12.5 for 75 min at 45°C. The numbers of alkali-labile lesions were calculated as \(-\ln(\text{fraction of DNA remaining in the CCC form after alkaline treatment})\). Results are given as means of three experiments ± SD.](https://academic.oup.com/carcin/article-abstract/17/2/327/269823/)
Table I. Effect of BLM, in combination with antioxidants, on the inactivation of PM2 DNA

<table>
<thead>
<tr>
<th>DNA exposure</th>
<th>Lethal hits</th>
<th>$P_{pt}$</th>
<th>DSB/lethal hits ($\times 100%$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLM 0</td>
<td>0</td>
<td>0</td>
<td>15±26</td>
</tr>
<tr>
<td>BLM 20 $\mu$M</td>
<td>0.5±0.1</td>
<td>0.07±0.01</td>
<td>15±26</td>
</tr>
<tr>
<td>+ 20 $\mu$M Asc</td>
<td>4.5±0.6</td>
<td>0.57±0.18</td>
<td>13±26</td>
</tr>
<tr>
<td>+ 200 $\mu$M Cys</td>
<td>4.2±0.9</td>
<td>1.26±0.17</td>
<td>31±22</td>
</tr>
<tr>
<td>+ 2000 $\mu$M GSH</td>
<td>1.4±0.4</td>
<td>0.38±0.10</td>
<td>30±16</td>
</tr>
<tr>
<td>+ 2000 $\mu$M NAC</td>
<td>1.4±0.4</td>
<td>0.65±0.17</td>
<td>51±28</td>
</tr>
</tbody>
</table>

pH 12.5 for 75 min at 45°C, were induced by BLM (see Figure 5). The alkali-labile lesions could only be measured as a decrease in CCC DNA. Alkali-labile lesions that may have been present in OC DNA (which by definition contain one or more SSB) could not be detected in this assay, since this DNA form is converted to single-stranded DNA fragments by alkali treatment. Unexposed DNA also appeared to contain alkali-labile sites, on average ~2 per PM2 DNA molecule. In the presence of GSH and Asc the number of these lesions was considerably reduced in both BLM-exposed and unexposed DNA. In the presence of Cys this reduction was less only without BLM. However, the number of alkali-labile lesions was not influenced by the presence of NAC.

Biological activity of the exposed bacteriophage DNA

The extent to which the PM2 DNA is still capable of forming mature virus particles (p.f.u.) is a measure of the biological activity of the DNA. From the exponential decrease in p.f.u. the mean number of lethal hits was calculated, summarized in Table I. Treatment of PM2 DNA with 20 $\mu$M BLM resulted in a clear biological inactivation, which was considerably increased when the exposure was performed in the presence of the thiol compounds or Asc. As is also shown in Table I, the biological inactivation can only partly be explained by the introduced DSB, which are known to be almost 100% lethal in PM2 DNA. In the case of exposure of PM2 DNA to BLM alone or BLM plus Asc the contribution of DSB to total inactivation is only ~15%. In the case of incubations in the presence of the thiol compounds this contribution varies from ~30% (Cys and GSH) to 50% (NAC). These results indicate that an important part of the DNA inactivation must be due to types of DNA damage other than DSB.

Discussion

The broad spectrum of its DNA damaging effects makes BLM a good model compound for studies of environment-related DNA damage (8). In order to induce DNA damage metal–BLM complexes need to be activated. For this activation process reducing agents, the presence of DNA and $O_2$ are required. Beside the direct genotoxic effects of BLM, generation of hydroxyl radicals is also important. The interaction between BLM and antioxidants is interesting from two different points of view: the combination of BLM with antioxidants may give (i) potentiation of DNA damage through their reducing properties, which may result in more effective treatment of cancer, or (ii) inhibition of DNA damage through scavenging and thereby protection of cells against environmental carcinogens or decreased side effects in a therapeutic approach in the case of anti-tumor treatment.

In the cell-free system used in this study the thiols NAC, Cys and GSH potentiated BLM-induced DNA damage. Moreover, the very potent antioxidant Asc, which was used for comparison, also increased BLM-induced DNA damage. The potentiating effect of the various compounds can be explained by the following sequence of reaction events. Cu$^{3+}$–BLM, the starting compound in our experiments (see Materials and methods) probably binds to DNA, followed by exchange of Cu ions chelated to BLM for Fe ions bound or chelated to DNA. That such an exchange takes place and that Cu$^{3+}$/Cu$^{+}$–BLM by itself is not able to damage DNA under our conditions was supported by the fact that when DNA was pre-treated with the strong Fe ion chelator desferal BLM no longer showed DNA damaging activity. Moreover, the activity of BLM was increasingly inhibited by increasing concentrations of Cu$^{3+}$ added to the reaction solution. After metal ion exchange most of the BLM bound to the DNA will be expected to be bound to Fe$^{3+}$. To become activated it is generally assumed that in the first step Fe$^{3+}$–BLM has to be reduced to Fe$^{2+}$–BLM by a reducing agent (18). Both the thiols used in our experiments and Asc can serve as reductants in this step. However, BLM also appears to have DNA damaging properties in the absence of a reducing agent (Figure 1). This can be explained by the assumption that Fe$^{3+}$–BLM can also to a small extent be activated by $O_2$ (the next activation step; see below) or that a small amount of the iron chelated with DNA is Fe$^{2+}$. In the next step of the activation process Fe$^{2+}$–BLM is converted by $O_2$ into ‘activated BLM’, which is probably a ferric peroxide: Fe$^{3+}$–HO$^-$–BLM. This latter species is highly oxidizing and able to abstract a H radical (‘H) from the DNA, in particular from C-4' of the deoxyribose moieties, which in turn in subsequent reactions lead to AP sites with an oxidized deoxyribose (alkali-labile sites) or to strand breaks with accompanying release of base propanals (19). That the presence of $O_2$ is a prerequisite for BLM activation and DNA damage can be determined from our finding that the introduction of DNA damage is strongly inhibited when air was replaced by $N_2$ (Figure 4). In addition to ‘H abstraction from deoxyribose, Fe$^{3+}$–HO$^-$–BLM can probably also decompose and eventually form OH radicals (‘OH), which are supposed to be mainly responsible for oxidative damage (9,20). Finally, Fe$^{3+}$–BLM, which is formed after reaction of Fe$^{3+}$–HO$^-$–BLM with DNA or decomposition, can be re-activated by reduction by reducing compounds and $O_2$, explaining the strong potentiating effects of the thiols and Asc that we have found in our experiments (21).

The advantage of the use of biologically active PM2 DNA is that the contribution of the various types of DNA damage to the total biological inactivation can be determined. Our experiments show that in the presence of the various antioxidants not only DNA damage, but also the degree of biological inactivation, is strongly enhanced. As shown in Table I, the contribution of DSB, which in PM2 DNA are lethal, to biological inactivation is rather low. In the absence of antioxidants this contribution is ~15%. A similar contribution was found in the presence of Asc, indicating that in this case the redox cycle reaction model described above fully applies. On the other hand, the contribution of DSB to lethality is higher in the case of the thiol compounds, ranging from, on average, ~30% in the presence of Cys and GSH to ~50% in the presence of NAC (Table I). This finding strongly suggests that the thiol compounds not only act as Fe$^{3+}$–BLM reducing agents, but, in addition, are somehow active in DNA damage formation leading to a shift from one type(s) of lethal damage to another (DSB).

We can only speculate on the other lesions which might be
Responsible for DNA inactivation. Important lesions formed as a consequence of reaction of activated BLM with DNA are alkali-labile (or AP) sites. Their presence could be established in the CCC form of PM2 DNA, but they probably represent only a small part of the total number of these lesions, since it is known that BLM-induced AP sites are found with a relatively high frequency in combination with closely opposed SSB and thus must be present in the OC form, which contains by definition at least one SSB (22,23). It is obvious that such double damage, e.g. SSB in combination with alkali-labile sites in the opposite strand or SSB or AP sites in similar combination with base damage, are very good candidates for lethal lesions other than DSB. After introduction of the exposed DNA into its bacterial host it can be expected that such double damage is converted to (lethal) DSB as a consequence of enzymatic DNA repair, which in most cases involves a DNA strand incision step.

The situation in our cell-free system may be different from that in cells exposed to BLM. It has been suggested that Cu\(^{2+}\)-BLM, which is quite stable to ligand substitution reactions, carries BLM to the nucleus, where Cu is replaced by Fe, possibly before BLM is bound to the DNA (24). It is obvious that if antioxidants interfere with Fe\(^{3+}\)-BLM before it becomes bound to DNA by scavenging reactive oxygen species ('OH) or by inactivating Fe\(^{3+}\)-BLM itself, antioxidants will exert a protecting effect. Recently Buettner and Mosely reported that antioxidants possess such a protecting activity by showing that free Fe\(^{3+}\)-BLM, in contrast to that bound to DNA, is inactivated by Asc to a 'redox inactive' form (21). The fact that we did not find such BLM inactivation in our experiments, in which BLM was pre-treated with Asc before addition of DNA, is probably due to the use of Cu\(^{2+}\)-BLM instead of Fe\(^{3+}\)-BLM in the Asc pre-treatment. Another important indication of protection (and potentiation!) by thiol of the clastogenic action of BLM in cells was obtained in studies in which a protective effect of Cys pre-treatment was found in human lymphocytes in vitro, whereas post-treatment with Cys led to potentiation (25).

In conclusion, according to our results the effect on BLM-induced DNA damage of antioxidants is mainly potentiating. This may increase the cytotoxicity of BLM as an anti-cancer drug. However, when we consider BLM as a model compound for various other carcinogens, supplementation with antioxidants as chemopreventatives may not always provoke the desired protective effect (26,27) and may depend on the subtle redox equilibrium within the cells.

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References


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