Journal of Environmental Pathology, Toxicology and Oncology, 32(3):189-193 (2013)

The Influence of Nonprotein Thiols on DNA Damage Induced by Bleomycin in Single Human Cells

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ABSTRACT: Nonprotein thiols are considered radioprotectors, preventing DNA damage by ionizing radiation. Because bleomycin (BLM) is a radiomimetic agent, it was proposed that thiols may prevent DNA damage produced by this antibiotic. However, results obtained with treatments combining thiols and BLM in living cells are contradictory. The goal of this study was to analyze the DNA damage induced by BLM and the influence of 3 nonprotein thiols of different electrical charges and chemical compositions at the level of single cells (comet assay). We also studied the morphological signs of apoptosis produced by BLM in these same conditions. We found that all thiols potentiated DNA damage induced by BLM, most probably by reactivating the BLM complex once it generated free radicals. Cysteamine (positive) potentiated BLM action the most, glutathione (negative) potentiated this antibiotic the least, whereas cysteine had an intermediate effect compared with the other two.

KEY WORDS: DNA damage, nonprotein thiols, comet assay

I. INTRODUCTION

Bleomycin (BLM) is a glycopeptide antitumor antibiotic that forms a complex with dioxygen and divalent metal ions (mainly Fe^{2+}), generating free radicals. It intercalates G-rich tracts of DNA and induces strand breakage by preferentially attacking pyrimidine nucleotides that adjoin the guanosyl-3-phosphate at the site of BLM-DNA binding.[1–4, 44] Several lines of evidence suggest that BLM action on the DNA of living cells is modulated by chromatin structure,[5–9] DNA repair,[6,8,10] degradation of BLM by BLM hydrolase,[11] antioxidant enzymes,[2,12,13] and thiol-containing compounds.[14–19]

Nonprotein thiols such as glutathione (GSH), are considered effective radioprotectors, preventing both DNA damage and cell death by ionizing radiation. BLM and other antibiotics such as enediynes are considered radiomimetic agents because they act through the generation of free radicals and produce chromosome damage in an S-independent manner.[20–23] This leads to the hypothesis that thiols may prevent DNA damage produced by these antibiotics.[14,16,19] However, results obtained by combining treatment with thiols and radiomimetic agents in naked DNA and in living cells are contradictory. Some studies showed a protective effect by thiols,[24–29] whereas others showed potentiation of DNA damage by antibiotics in the presence of the same thiols.[26,30–35] These discrepancies have been explained in terms of the amounts of oxygen and other different components of the cell culture media.[16,24,33] On the other hand, the ability and magnitude of protection of thiols compounds can be determined by its net charge[12,14,15]and the amount of its amine groups.[14] The hydroxyl radical generated by the BLM complex abstracts a hydrogen in the 4′ position of the deoxyribose. Once free radicals are produced, the Fe^{+2} in the BLM complex is oxidized to Fe^{+3}, and BLM becomes inactivated.[2,4] Thiols can reduce the deoxyribose molecule by donating a proton, inhibiting all subsequent oxidative DNA damage—a pro-

cess called "chemical repair."[14,15] In addition, thiols can reactivate the BLM complex by reducing the iron to the Fe^{+2} forms.[14,15] A balance between these 2 processes can determine the net effect of thiols on DNA damage induced by BLM.

We had previously found that at the chromosomal level and during cell death, GSH, β-mercaptoethanol, and cysteine (CYS) showed a protective effect, whereas at the DNA level all thiols potentiated the DNA damage induced by BLM, most probably because of a reactivation of the BLM complex. Cell survival and DNA repair also were improved by GSH, CYS, and cysteamine (CSM) (Mira et al., 2013 unpublished data).

The goal of this work was to analyze (by comet assay) the DNA damage induced by BLM and the influence of 3 nonprotein thiols with a different electrical charge and chemical composition at the level of single cells. We also studied the morphological signs of apoptosis produced by BLM in the same conditions as above using dichlorohydrate-4′, 6-diamine-2-phenylindol [DAPI] stain assay.

II. MATERIALS AND METHODS

Human lymphoblastoid cells (T-37 cell line, obtained from the IMBICE Cell Repository) were grown in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a 5% carbon dioxide atmosphere.

Cells were treated with BLM (100, 125, 150, 175, or 200 μg/mL) for 15 minutes (Lab. Gador, Argentina, Cultural Abstract Service [CAS] no. 9041-93-4). GSH (CAS no. 70-18-8), CYS (CAS no. 7048-04-6), and CSM (2-aminoetanethiol; CAS no. 156-57-0) were the thiols used in this study (all from Sigma Chemical Co.). Thiol compounds (10 mM) were added to the cultures 0.5 hours before BLM and left until the end of the BLM treatment (pretreatments) or immediately after the end of BLM treatment and left in the culture medium until harvesting (posttreatments). At the end of the pulse treatment with BLM, the cells were washed twice with Hank's balanced salt solution and kept in culture with fresh culture medium until harvesting. Buthionine sulfoximine (BSO) reduces intracellular GSH by about 95%. Therefore, we used a sample treated with BSO as an additional control. Single-strand DNA breaks and alkaline labile sites (converted to single-strand DNA breaks by alkaline buffer) were estimated by comet assay (alkaline single-cell gel electrophoresis).Comet assay was performed according to the method described by Singh et al.,[36] with some minor modifications. About 15 μL of cells were mixed with 75 μL of low-melting agarose 0.5% (Gibco BRL, Grand Island, NY), seeded on a slide coated with 0.5% normal-melting agarose (Promega, Fitchburg, WI), and cooled until solidified (2 different slides per treatment). After work, the cells were lysed overnight in a detergent solution (100 mM EDTA, 2.5 M sodium chloride, 10 mM Tris, 1% Triton X-100, and 10 % dimethyl sulfoxide).

Before electrophoresis, the slides were equilibrated in alkaline electrophoresis solution (1 mM EDTA, 300 mM sodium hydroxide, pH >13) for 20 minutes. Electrophoresis was carried out for 30 minutes at 25 V and 300 mA (1.25 V/cm). Then, slides were neutralized by washing them 3 times with Tris buffer (pH 7.5) every 5 minutes and distilled water.

Slides were stained with 35 μL ethidium bromide (0.1 μg/mL). The analysis was performed using fluorescence microscopy. We counted the first 200 nuclei in each treatment. According to the comet tail, 5 grades were assigned, from 0 (no tail) to 4 (detectable head but most DNA in the tail).[37] The damage index (DI) was calculated as follows: $DI = (n1 + 2n2 + 3n3 + 4n4)/N$, where *n1* is the number of cells in grade 1, *n2* is the number of cells in grade 2, *n3* is the number of cells in grade 3, *n4* is the number of cells in grade 4, , and N is the total number of cells analyzed per slide.[38] Images were captured with a Sony charge-coupled device camera and saved using Pro Plus imaging software.

Using a BLM concentration response curve we chose a concentration that produced mainly grade 2 comets (see Results). Cells were treated with the above-mentioned dose concentration before or after treatment, with 10 mM of each thiol employed

during 30 minutes.

Nuclear morphology and cell viability also analyzed. Cells were fixed with 4% paraformaldehyde stained with 10% DAPI and analyzed by fluorescent microscopy (using the same microscope and filters as those used for the comet assay). Cell viability was determined using the dye exclusion (trypan blue) method. Because trypan blue does not enter apoptotic cells but only death cells (necrotic cells) and because apoptosis needs more time than that used in these exclusion experiments, it can be assumed that the percentage of death cells are equivalent to the percentage of necrotic cells.

For comet assay we analyzed the data using analysis of variance. For morphological signs of apoptosis by DAPI stain we analyzed the results of the BLM combination with the different thiols using Poisson regression analysis. In this test, negative coefficient values indicate a protective BLM effect, whereas positive values indicate potentiation of the BLM damage. Three experiments of each type (comet assay, trypan blue exclusion, and apoptosis analysis) were carried out. Results are expressed as means ± standard errors (SEs).

III. RESULTS

For analysis of comet assay (see Fig. 1) 200 nuclei per treatment were analyzed and the DI was calculated. Four independent experiments were performed. Data are shown as mean ± SE. BLM induced a dose-dependent increase in DI compared to control samples ($P < 0.0001$) (Fig. 2). Taking into account that BLM 150 µg/mL showed a significant increase of DI (mean ± s.e = 1.29 ± 0.15) with respect to the control sample (mean ± s.e. = 0.28 ± 0.06), we chose to use this concentration in the combined treatments with thiols. At higher doses, samples showed too much DNA damage, with a big increment of grades 3 and 4.

Combined treatment produced more damage than BLM alone ($P < 0.0001$). When thiol treatments were made after the addition of BLM, DNA damage was higher than when they were added before BLM ($P < 0.0001$) (Fig. 3). Treatments with CYS and CSM, both before and after BLM, increase the DI the most. When GSH was depleted by BSO there was not a significant change with respect to BLM alone ($P < 0.0736$) (Fig. 4). The DI of BSO and BLM was lower than the DI of GSH and BLM ($P < 0.0163$).

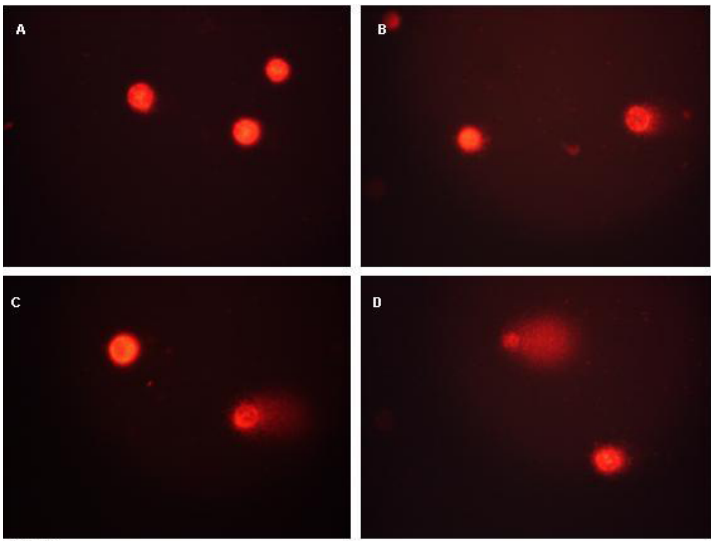

FIG. 1: Images of comets obtained in isolated lymphoblastoid cells using the alkaline version by Singh et al.[36] **A:** Nuclei showing no damage. **B:** Nuclei showing a small DNA migration corresponding to grades 1 and 2. **C, D:** Nuclei in which the typical shape of comet tails (grades 3 and 4) is observed.

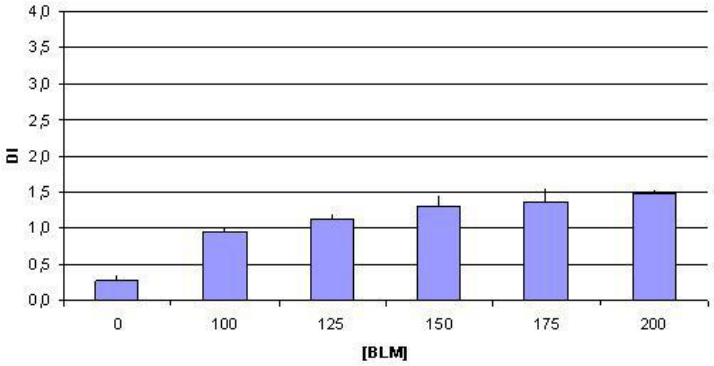

FIG. 2: Analysis of DNA damage (damage index [DI]) induced by bleomycin (BLM) (0–200 µg/mL) by comet assay. Data are means ± standard errors.

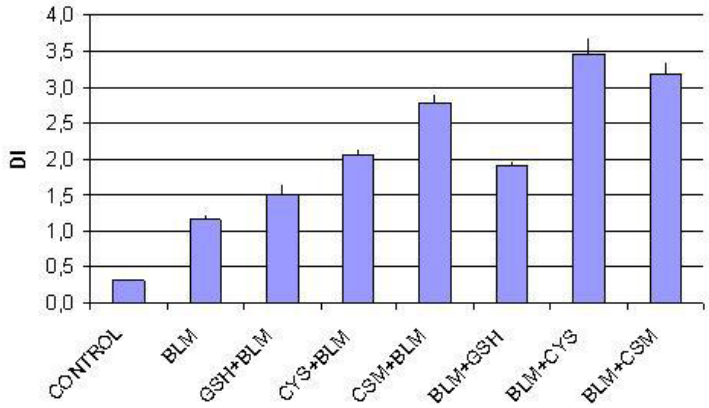

FIG. 3: Analysis of DNA damage (damage index [DI]) induced by bleomycin (BLM) (150 µg/mL) and with the following thiols before or after treatment: glutathione (GSH), cysteine (CYS), and cysteamine (CSM). Data are means ± standard errors.

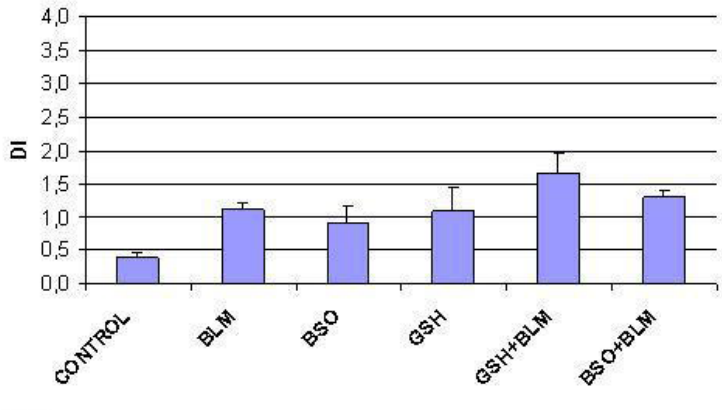

FIG. 4: Analysis of DNA damage (damage index [DI]) induced by bleomycin (BLM) (150 µg/mL) and before treatment with buthionine sulfoximine (BSO) and glutathione (GSH). Data are means ± standard errors.

Cell viability and apoptotic nuclear morphology were assayed 24 hours after treatment with BLM. Cell viability diminished when BLM concentration increased (0–200 μg/mL) ($P < 0.0001$), as shown by the dye exclusion (trypan blue) method.

For the combined treatments (BLM plus thiols; BLM plus BSO), we used the same dose as that used for the comet assay (BLM 150 μg/mL) (Fig. 5). All treatments (BLM, BLM plus thiols, thiols plus BLM, BSO plus BLM) diminished cell survival compared to control samples. Compared to BLM alone, only GSH added after BLM produced an increase of cell survival (about 20%; $P < 0.0444$). The rest of the combined treatments did not produce any significant change in cell survival compared to BLM alone (Fig. 5)

DAPI stain assay for nuclear imaging in fluorescence microscopy was used to analyze morphological signs of apoptosis (see Fig. 6). Morphological analysis revealed a dose-dependent formation of apoptotic bodies up to 175 μg/mL. Afterward, the curve of apoptotic cells reaches a plateau (BLM 200 μg/mL) ($P < 0.0001$).

For apoptosis analysis with combined treatments, BLM 150 μg/mL also was used (as in the comet and cell viability assays). When CYS was added after BLM treatment, apoptosis was lower than the apoptosis produced by BLM alone ($P < 0.005$). However, when CYS was added before BLM, apoptosis increased ($P < 0.0001$). GSH added before BLM also increased apoptosis ($P < 0.025$) (Fig. 7). The rest of thiols did not produce any significant changes in apoptosis compared to BLM alone. CYS before BLM treatment increased

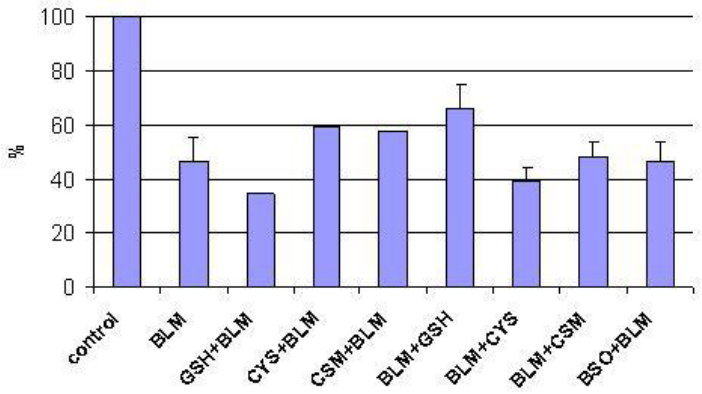

FIG. 5: Analysis of cell survival 24 hours after incubation with bleomycin (BLM) (150 μg/mL) and with the following compounds before or after treatment: glutathione (GSH), cysteine (CYS), cysteamine (CSM), and buthionine sulfoximine (BSO). Data are means ± standard errors.

FIG. 6: Images of nuclear morphology of apoptotic (**A, B**) and nonapoptotic (**C**) lymphoblastoid cells. **A** and **B** show one nucleus with fragmentation. On the other hand, **C** shows 2 different nuclei with no fragmentation.

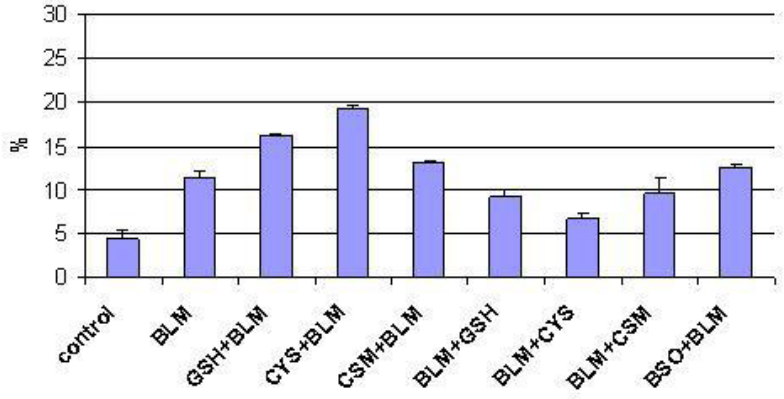

FIG. 7: Analysis of apoptosis 24 hours after incubation with bleomycin (BLM) (150 µg/mL) and with the following compounds before or after treatment: glutathione (GSH), cysteine (CYS), cysteamine (CSM), and buthionine sulfoximine (BSO). Data are means ± standard errors.

apoptosis much more than GSH did before BLM treatment (Poisson regression coefficients: CYS plus BLM = 0.52; GSH plus BLM = 0.34) (Fig. 7). BSO did not produce any change in the amount of apoptotic cells compared to BLM alone (Fig. 7).

IV. DISCUSSION

DI analysis showed that all thiols potentiated DNA damage induced by BLM. The influence of thiols on the effect of BLM on DNA may be due to (1) chemical repair of the deoxyribose molecule by the donation of a hydrogen from the thiol, (2) reactivation of the BLM complex by reducing Fe^{+3} to Fe^{+2}, and (3) free radical scavenging. Because all thiols potentiated the damage, event 1 did not occur or it was of lesser importance. Even free radical scavenging can occur (event 3), but our data suggest that reactivation of BLM complex (event 2) by thiols is the most important event explaining our results. Other studies have demonstrated that thiols' ability to access DNA is determined by electrostatic interactions between the net change of the thiol and the negative charge of the DNA.[17] The final result is that positive thiols (CSM) have better access to DNA than neutral ones (CYS, which is actually a Zwitterion), whereas negative thiols (GSH) have restricted access. Once the BLM complex generates free radicals the iron in that complex is oxidized (from Fe^{+2} to Fe^{+3}). Because thiols are hydrogen donors they can reduce the iron (from Fe^{+3} to Fe^{+2}), reinitiating the damage by BLM.[39]

As an amine, CSM binds to DNA,[16,32,33,35] and it may alter DNA conformation so as to facilitate BLM action. The widening of the minor groove of DNA (which is the likely site of BLM binding[2,14,18,40–42]) by the amine group may give BLM better access to its target. Rather than being alternatives, the thiol and amine functions may act in concert.[16]

CYS produced more damage than CSM (CSM as a positive thiol should produce more DNA damage than CYS, which lacks the positive charge). However, after treatment with these 2 thiols we observed that CYS had a higher DI. This is not consistent with the above-mentioned properties of CSM, so we decided to take a look at the damage in each DNA comet grade (Fig. 8). In this way we observed that the only case in which CYS produced more damage than CSM was in grade 4 of the post-treatments. Grade 4 is multiplied by 4, therefore giving more relevance to the DI if the number of cells in this grade is high, as it is in this case. Although in this experiment the mentioned grade was unusually high for CYS and CSM, we had to take into account that the fragmentation of DNA in this grade was not only due to DNA breaks induced by BLM but also because most of the DNA frag-

ments in grade 4 corresponded to pieces of DNA produced by cellular necrosis and apoptosis. Then, we analyzed the number of cells in grades 2 and 3 (whose DNA fragmentation is mainly produced by BLM action), and we observed that CSM had more cells than CYS in these grades. Therefore, it is reasonable to assume that in this case CSM produced more damage than CYS. We checked the differences between grades 2 and 3 for all thiols using a Poisson regression analysis (multivariate analysis). These differences between CSM and CYS were highly significant ($P < 0.01$).

When we used BSO (which eliminates most GSH), the DI was similar to that of BLM alone. Another study in which GSH and BLM were used simultaneously demonstrated that the presence of this thiol potentiates the clastogenic action of BLM in mammalian lymphocytes. This was attributed to GSH acting as a reducing agent in reactivating the oxidized BLM.[30,39,43] In addition, it was shown that BLM treatment to BSO-treated cells reduced the frequency of aberrant metaphases and chromatid breaks.[39] This reduction in the effect of BLM in GSH-depleted cells might be explained by the failure of reactivation of the oxidized BLM by the reducing agent (GSH), which was present endogenously.[41]

Thiol posttreatments potentiated the DNA damage by BLM more than thiol pretreatments, probably because of an enhanced reactivation of the BLM complex.[44]

Although previous treatments with thiols did not show changes in cell survival at 24 hours compared to BLM alone, other studies from our laboratory (data not shown) using BLM 100 µg/mL and the same thiols showed similar results at 24 hours but almost total killing (15%) at 96 hours. In other words, the results observed at 24 hours would be at a transient state of the cells, which would die at 96 hours. The damage observed immediately after BLM treatment, which is not reflected in cell survival at 24 hours, might be due to DNA repair occurring during the lapse between the moment we checked DNA damage and the moment we counted surviving cells. Only GSH posttreatments increased cell viability, although this thiol potentiated the DNA damage the least. GSH, being negative, would have a restricted access to DNA, but it could reduce the oxidative stress produced by BLM, hence improving cell survival. GSH probably would work as a cofactor of dismutase superoxide and would stabilize repair proteins so that it could prevent the action of reactive oxygen species. GSH can also increase DNA repair.

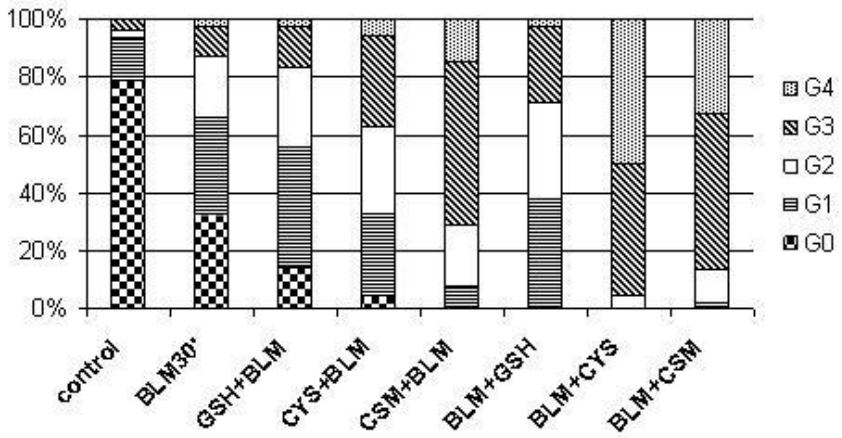

FIG. 8: Analysis of comet assay grades for each treatment (control, bleomycin [BLM], glutathione [GSH] + BLM, cysteine [(CYS] + BLM, cysteamine [CSM] + BLM, BLM + GSH, BLM + CYS, and BLM + CSM). Each bar represents the percentages of grades (G) 0–4 produced by the respective treatments.

It has been shown that GSH affects DNA double-strand breaks, rejoining, and exchanges.[39,45,46] In this way, GSH can act by either reactivating the BLM complex or scavenging free radicals, depending on cell conditions. A similar phenomenon was attributed to CSM. Other studies showed that CSM has a protective effect under hypoxia in the culture medium. Under oxygenated conditions, on the other hand, CSM loses its protective effect on DNA.[16,24,25,27,32,33,47]

Although thiol posttreatments increased DNA damage by BLM, this was not correlated with cell survival and apoptosis. A great part of the DNA damage induced by the antibiotic are DNA single-strand breaks that can be totally repaired during the 24 hours from the moment cell viability and apoptosis were analyzed. It can explain the discrepancy among DIs, cell survival, and apoptosis.

Previous treatments with GSH and CYS produced an increase of apoptosis. This phenomenon may induce a high oxidative stress that, in turn, can give rise to apoptosis. It was shown that reactive oxygen species can act as a signal for the induction of apoptosis.[48–57] Because CYS is partly converted within the cell in GSH, the decrease in apoptosis by CYS posttreatments might be due to the above-mentioned free radical scavenging and enzymatic DNA repair (i.e., GSH can stabilize DNA repair proteins and be a cofactor of superoxide dismutase).

Free radicals generated by BLM are produced in a chromatin microdomain. Such radicals are highly unstable and react with organic macromolecules that are near to the BLM intercalation site. Because thiols are free radical scavengers, a competence between these thiols and DNA (for the free radicals) might occur.[4,58]

Our methods led us to conclude that DNA damage induced by BLM plus CSM was higher than that produced by BLM plus CYS, even though DI showed the opposite (i.e., combined treatment with CYS was higher than combined treatment with CSM). Therefore we suggest that it is convenient to analyze the number of cells in grades 2 and 3 because they mostly reflect strand breaks induced by BLM (and other clastogenic agents).

These experiments and the ones conducted in this report can help us better understand the significance of the interaction between thiols and BLM, information that is of clinical interest since this antibiotic is routinely employed to treat several illnesses.

ACKNOWLEDGMENTS

This work was supported by grants from CONICET (PIP 03001), ANPCyT (PICT 14329), and the CIC of Argentina. The authors thank César Horgan and Verónica Labarta for technical assistance and Alejandro Portela, from Gador Laboratories of Argentina, for providing the bleomycin.

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