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Anabela Mira PhD [a b], Esteban M. Gimenez PhD [a], Alejandro D. Bolzán PhD [a c], Martha S. Bianchi PhD [a c] & Daniel M. López-Larraza PhD [a c]

[a] Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CCT-CONICET La Plata-Comisiôn de Investigaciones Cientificas de la Provinincia de Buenos Aires), La Plata, Argentina

[b] Becaria de la Agencia Nacional de Promociôn Cientifica y Tecnolôgica (ANPCyT), Buenos Aires, Argentina

[c] Carrera del Investigador Cientifico del Consejo Nacional de Investigaciones Cientificas y Tècnicas, Argentina. (CONICET).

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Effect of Thiol Compounds on Bleomycin-Induced DNA and Chromosome Damage in Human Cells

Anabela Mira, PhD; Esteban M. Gimenez, PhD; Alejandro D. Bolzán, PhD; Martha S. Bianchi, PhD; Daniel M. López-Larraza, PhD

ABSTRACT. Non-protein thiols are considered radioprotectors, preventing DNA damage by ionizing radiation. As bleomycin (BLM) is a radiomimetic agent it was proposed that thiols may prevent DNA damage produced by this antibiotic. However, results obtained with thiols and BLM-combined treatments in living cells are contradictory. The goal of this work was to assess the influence of five non-protein thiols of different electrical charge and chemical composition, on the DNA damage, DNA repair, chromosomal aberrations and cell killing induced by BLM. We found that, at the chromosomal level and cell killing, Glutathione, β-Mercaptoethanol and cysteine showed a protective effect, while ditiothreitol and cysteamine increased them, whereas at the DNA level all thiols potentiated the DNA damage induced by BLM, most probably due to a reactivation of the BLM complex.

KEYWORDS: bleomycin, chromosomal aberrations, DNA damage, thiols

Bleomycin (BLM) is a glycopeptide antitumor antibiotic that complexes with dioxygen and divalent metal ions (mainly Fe^{2+}), generating free radicals. It intercalates G-rich tracts of DNA and induces strand breakage by preferential attacking of pyrimidine nucleotides that adjoin the guanosyl-3-phosphate at the site of BLM-DNA binding.[1,2] Misjoining of double-strand DNA breaks gives rise to chromosomal aberrations.[3]

Several lines of evidence suggest that the BLM action on the DNA of living cells is modulated by chromatin structure,[4–6] DNA repair,[5–7] degradation of BLM by BLM hydrolase,[8] antioxidant enzymes,[9,10] and thiol-containing compounds.[11–15]

Nonprotein thiols, as glutathione (GSH), are considered effective radioprotectors, preventing both DNA damage and cell killing by ionizing radiation. BLM and other antibiotics such as the enedyines are considered radiomimetic agents due to the fact that they act through the generation of free radicals and produce chromosome damage by an S-independent manner.[16–18] This leads to the hypothesis that thiols may prevent DNA damage produced by these antibiotics.[12,13] However, results obtained with thiols and radiomimetic agents–combined treatments in naked DNA and in living cells are contradictory. Some studies showed a protective effect by thiols,[19–24] whereas others showed potentiation of DNA damage by antibiotics in the presence of the same thiols.[12,19,25–30] These discrepancies have been explained in terms of the amounts of oxygen and other different components of the cell culture media.[12,20,30] On the other hand, it has been observed that the net charge[9,13,14] and the amount of amine groups[13] of thiols can determine their ability and the magnitude of the protection conferred by these compounds.

Anabela Mira, **Esteban M. Gimenez**, **Alejandro D. Bolzán**, **Martha S. Bianchi**, *and* **Daniel M. López-Larraza** *are from the Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CCT-CONICET La Plata–Comisiôn de Investigaciones Cientîficas de la Provinincia de Buenos Aires), La Plata, Argentina*. **Anabela Mira** *is also a fellowship of the Becaria de la Agencia Nacional de Promociôn Cientîfica y Tecnolôgica (ANPCyT), Buenos Aires, Argentina.* **Alejandro D. Bolzán**, **Martha S. Bianchi**, *and* **Daniel M. López-Larraza** *are members of the Carrera del Investigador Científico del Consejo Nacional de Investigaciones Cientîficas y Tècnicas, Argentina (CONICET).*

The hydroxyl radicals generated by the BLM complex abstract a proton 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. However, activated BLM is probably BLM-Fe(III)-OOH, and it may directly (or indirectly) remove the 4' hydrogen.[1] Thiols can reduce the deoxyribose moiety by donating a proton, inhibiting all subsequent oxidative DNA damage, a process called "chemical repair."[13,14] On the other hand, thiols can activate (first event on the BLM activation) and reactivate (reactivation of BLM once it produced the damage on the DNA) BLM complex by reducing the iron to the Fe^{2+} forms.[13,14] A balance between these 2 processes can determine the net effect of thiols on DNA damage induced by BLM.

The goal of this work was to assess the influence of 5 nonprotein thiols of different electrical charge and chemical composition, on the DNA damage, DNA repair, chromosomal aberrations, and cell killing induced by BLM.

We found that, at the chromosomal level and cell killing, GSH, β-Mercaptoethanol (BME), and cysteine (CYST) showed a protective effect, whereas at the DNA level all thiols potentiated the DNA damage induced by BLM, most probably due to a reactivation of the BLM complex. Cell survival and DNA repair were also improved by GSH, CYST, and BME.

METHODS

Culture conditions and treatment

Human lymphoblastoid cells (T-37 cell line, obtained from the IMBICE Cell Repository, La Plata, Buenos Aires, Argentina) were grown in RPMI 1640 medium (Sigma Chemical, St. Louis, MO, USA) supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in 5% CO_2 atmosphere. Cells were seeded in TC25 Corning flasks at a density of 6×10^5 cells/mL of culture medium. Cells were seeded in such a concentration that at 24 hours were at a log phase of growth, time at which the appropriate treatment was carried out (for the analysis of chromosome-type aberrations) or the last 5 hours of cell culture (for the analysis of chromatid-type aberrations induced in the G2 phase of cell cycle). The cells were treated with BLM (CAS No. 9041-93-4; Lab. Gador, Ciudad Autônoma de Buenos Aires, Buenos Aires, Argentina) at the time and concentrations indicated in the figures. Time of exposure and concentration of BLM used in the cytogenetic experiments were chosen according to previous experience in our laboratory (Bolzán, unpublished, data not shown). Thiols used in this work (all from Sigma Chemical) were glutathione (GSH; CAS No. 70-18-8), β-mercaptoethanol (BME; CAS No. 60-24-2), dithiothreitol (DTT; CAS No. 3483-12-3), Cysteine (CYST; CAS No. 7048-04-6), and cysteamine (= 2-aminoetanethiol) (CSM; CAS No. 156-57-0). Thiol compounds (0.5 to 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 Hanks' balanced salt solution and kept in culture with fresh culture medium until harvesting.

Cell harvesting and cytogenetic analysis

For the cytogenetic analysis, cells were always harvested 24 hours after the end of treatments. To determine the effect of thiols on the chromosome induced by BLM throughout the interphase and specifically in the G2 phase of cell cycle, T-37 cells were treated with BLM during the log phase of growth (Figure 1) or in the last 5 hours of cell culture (Figure 7), respectively (time of exposure and concentrations indicated in the figures). At the end of the pulse treatment with BLM, the cells were washed twice with Hanks' balanced salt solution and kept in culture with fresh culture medium containing 5-bromo-2'-deoxiuridine (BrdU) until harvesting.

During the last 3 hours of culture, the cells were exposed to colchicine (0.1 mg/mL) (CAS No. 64–86-8; Sigma). Chromosome preparations were made according to standard procedures and stained with Giemsa. In all cases, 100 metaphases per treatment and per experiment were scored for aberration analysis. The aberrations scored included dicentrics, rings, deletions or acentric fragments, chromatid and chromosome breaks, and chromatid exchanges.

Chromosome analysis was restricted to first mitosis cells. This was confirmed by staining one slide from each sample with a modification of the fluorescence plus Giemsa (FPG) method.[39] Briefly, slides were stained with Hoechst 33258 (1 μg/mL) (CAS No. 23491–45-4; Sigma) in 0.1 M

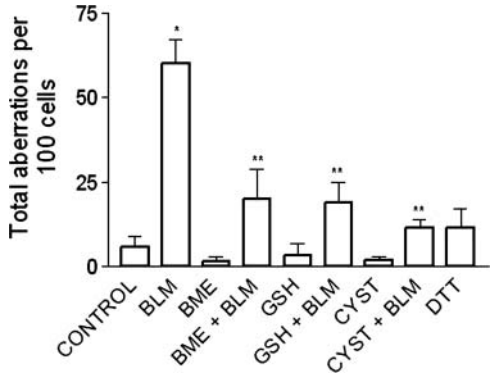

Fig. 1. Effect of thiol compounds on BLM-induced chromosome damage (100 μg/mL, 2 hours, 37°C) in T-37 cells—Pretreatments (I). Thiols: 10 mM, 30 minutes before BLM treatment. Harvesting time: 24 hours after treatments. Data represent average values of 2 independent experiments (mean ± *SEM*); 100 cells per sample per experiment analyzed. No metaphase cells were found in CSM ± BLM and DTT + BLM treatments. *$p < .05$ compared with control. **$p < .05$ compared with BLM alone.

phosphate buffer (pH 6.8) for 30 minutes, exposed to ultraviolet (UV) light (365 nm) for 1 hour, and stained with Giemsa.

Mitotic index (MI)

The MI was determined in 1,000 cells and expressed as the percentage of cells in mitosis.

Cell proliferation kinetics

The cell proliferation kinetics was defined as the proportion of first (M1), second (M2), and third (M3) mitotic divisions and it was determined by scoring 100 metaphases per sample per experiment. First-, second-, and third-division metaphases were identified using differential chromatid labeling.[31] Cell proliferation kinetics was expressed as the replication index (RI), which was calculated according to Ivett and Tice[32] by the formula:

$$RI = [M1 + 2(M2) + 3(M3)]/100$$

Low-voltage agarose electrophoresis to estimate DNA damage and repair

High-molecular-weight DNA from control, BLM-treated (100 μg/mL, 15 or 45 minutes), and combined-treated (thiols plus BLM) cells were extracted with standard protocols. Double-stranded and single-stranded DNA degradation was assayed by neutral and alkaline agarose gel electrophoresis, respectively. The DNA samples (1 μg) were separated in 0.6% agarose gels at 2 V/cm, for 5 hours. Neutral gels were stained with 0.5 μg/mL ethidium bromide in distilled water. Alkaline gels were stained for 40 minutes with Syber Green II (Invitrogen, Carlsbad, CA, USA) (as recommended by the manufacturer) after neutralization in 1 M Tris-HCl, 1.5 M NaCl, pH 8.0. Stained gels were digitally photographed and analyzed with Kodak Science 1 D program.

Cell survival analysis

Control (no BLM, no thiols), BLM-treated (100 or 500 μg/mL, 15 minutes or 2 hours), and combined-treated (BLM and pre- and posttreatment with thiols, all at 10 mM during 0.5 hour) cells were seeded in 24-well plates at a concentration of 3×10^5/mL. After 96 hours, aliquots were stained with trypan blue and the percentage of cell survival was scored. In some experiments, cells survival was determined at 24, 48, 72, and 96 hours. As employed doses were cytotoxic, we determine the lethal concentration fifty (LC_{50}), which is the concentration of BLM producing the killing of 50% of the exposed cells. Cells were treated with increasing concentrations of BLM ranging from 0.1 to 300 μg/mL. Using the Graph Pad Prism 3.02 program (Graph Pad Software, San Diego, CA, USA), the best-fitting curves (as estimated by the R^2) were built and the LC_{50} was determined by interpolation on the x-axis. Then, we treated cells as described before, but using the LC_{50} dose alone or in combination with thiols.

Statistical analysis

The significance of differences in aberration frequencies among different treatments was obtained by 1-way analysis of variance (ANOVA). The level of significance chosen was $p < .05$. The LC_{50} for survival experiments was determined by the best-fitting curve. Differences in survival among different treatments were analyzed as for differences in chromosomal aberrations. The statistical analysis of chromosome aberrations data was based on absolute numbers of aberrations per 100 cells per sample. In all cases we used Prism 3.03 software (Graph Pad Software) (licensed to Daniel Lopez-Larraza).

RESULTS

Chromosomal aberrations

The effect of thiols (10 mM) added 30 minutes before BLM treatment on the yield of BLM-induced chromosomal aberrations (CAs) in T-37 cells is shown in Figure 1. A statistically significant increase in the frequency of CAs was observed in T-37 cells following treatment with BLM ($p < .05$) (Figure 1). Treatments with BME, GSH, CYST, and DTT alone did not produce any significant increase of CAs over control values ($p > .05$; Figure 1), whereas CSM alone resulted cytotoxic for T-37 cells. The addition of BME, GSH, and CYST produced a significant decrease in the yield of chromosome damage induced by BLM, whereas no metaphases were found in the combined treatments BLM + CSM and BLM + DTT, which prevented the cytogenetic analysis. In order to confirm the effect of CSM and DTT alone or in

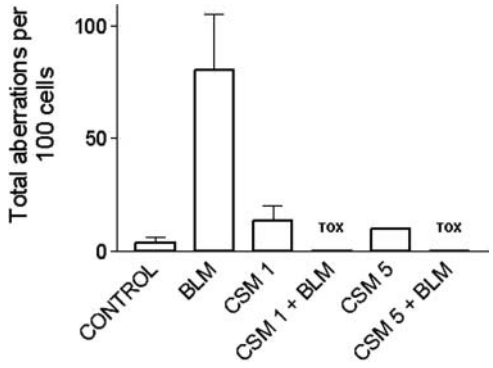

Fig. 2. Effect of CSM on BLM-induced chromosome damage (100 μg/mL, 2 hours, 37°C) in T-37 cells—Pretreatments (II). Cysteamine (CSM): 1 and 5 mM, 30 minutes before BLM treatment. Harvesting time: 24 hours after treatments. Data represent average values of 2 independent experiments (mean ± *SEM*); 100 cells per sample per experiment analyzed. TOX: No metaphase cells were found in CSM + BLM treatment, because it was cytotoxic.

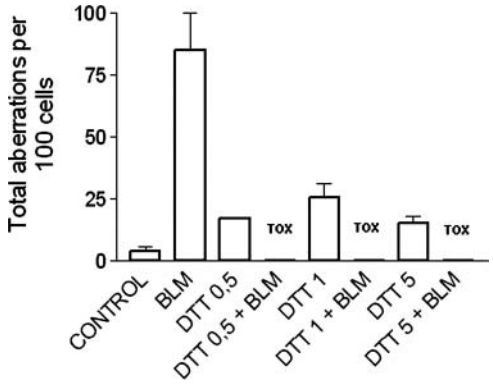

Fig. 3. Effect of DTT on BLM-induced chromosome damage (100 μg/mL, 2 hours, 37°C) in T-37 cells—Pretreatments (III). DTT: 0.5, 1, and 5 mM, 30 minutes before BLM treatment. Harvesting time: 24 hours after treatments. Data represent average values of 2 (DTT 5 mM) or 3 (DTT 1 mM) independent experiments (mean ± *SEM*). Only one experiment was performed with DTT 0.5 mM; 100 cells per sample per experiment analyzed. TOX: No metaphase cells were found in DTT + BLM treatment, because it was cytotoxic.

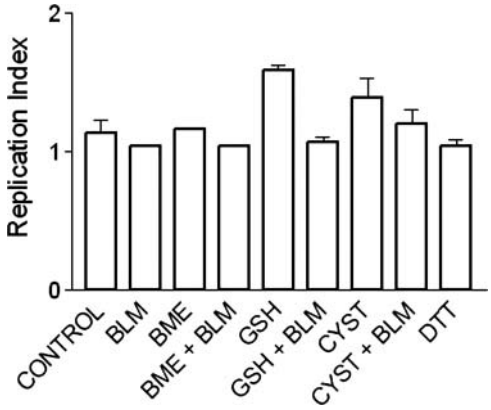

Fig. 5. Effect of thiol compounds ± BLM (100 μg/mL, 2 hours, 37°C) on replication index in T-37 cells. Thiols: 10 mM, 30 minutes before BLM treatment. Harvesting time: 24 hours after treatments. Data represent average values of 2 independent experiments (mean ± *SEM*); 100 cells per sample per experiment analyzed. No metaphase cells were found in CSM ± BLM and DTT + BLM treatments.

combination with BLM in T-37 cells, we carried out 2 additional experiments, using lower doses of these thiols (Figures 2 and 3). These experiments showed that whereas CSM at doses lower than 10 mM is not clastogenic per se, DTT alone is clastogenic, and both of them actually potentiate the cytotoxicity and clastogenicity of BLM on T-37 cells, as shown by the absence of metaphases or the presence of a few of them, all exhibiting severe chromosome damage in the combined treatments. Further analysis showed that BLM alone produced a significant decrease in the mitotic index compared with control cultures. BME + BLM showed a significant decrease respect to BLM alone ($p < .05$), whereas the other thiols did not show any significant differences (Figure 4). Moreover, no significant differences were observed between the replication index of BLM alone–treated cultures and those treated with BLM and thiols (Figure 5).

When thiols were added after BLM treatment, none of them showed any significant differences on the clastogenic

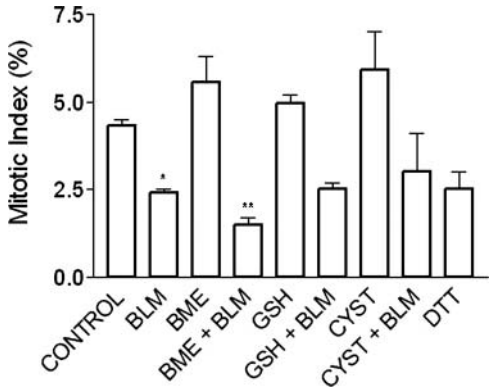

Fig. 4. Effect of thiol compounds ± BLM (100 μg/mL, 2 hours, 37°C) on mitotic index in T-37 cells—Pretreatments (I). Thiols: 10 mM, 30 minutes before BLM treatment. Harvesting time: 24 hours after treatments. Data represent average values of 2 independent experiments (mean ± *SEM*); 1,000 cells per sample per experiment analyzed. No metaphase cells were found in CSM ± BLM and DTT + BLM treatments. *$p < .05$ compared with control. **$p < .05$ compared with BLM alone.

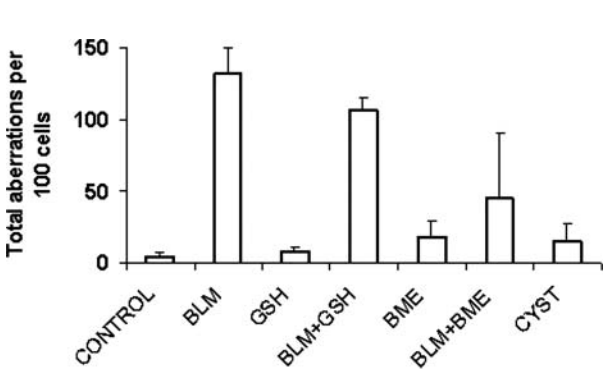

Fig. 6. Effect of thiol compounds on BLM-induced chromosome damage (100 μg/mL, 2 hours, 37°C) in T-37 cells—Postreatments. Thiols: 10 mM, during 24 hours after BLM treatment. Harvesting time: 24 hours after treatments. Data represent average values of 3 independent experiments (mean ± *SEM*); 100 metaphase cells per sample per experiment analyzed. No metaphase cells were found in BLM + CYST treatment.

action of the antibiotic (Figure 6), whereas no metaphases were found in the combined treatments BLM + CYST. Posttreatments with CSM and DTT showed that CSM at the concentrations of 0.5 and 10 mM is cytotoxic, whereas DTT alone is moderately clastogenic at the concentration of 0.5 mM and cytotoxic at the concentration of 10 mM, resulting in the absence of metaphases or the presence of a few of them, all exhibiting severe chromosome damage in these treatments (data not shown). No metaphases were found in the combined treatments BLM + CSM and BLM + DTT (irrespective of the concentrations used), which prevented the cytogenetic analysis (data not shown). Moreover, analysis of the mitotic index showed similar results to those obtained in the pretreatment experiments, that is, a lower mitotic index in BLM alone–exposed cells in comparison with control cultures, and no significant differences between combined treatments and BLM-treated cells (data not shown). Again, no significant differences were observed between the replication index of BLM alone–treated cultures and those treated with BLM and thiols (data not shown).

On the other hand, none of the thiols had a significant effect on the yield of chromatid-type aberrations induced in the G2 stage of cell cycle by BLM in T-37 cells (Figure 7). Analysis of the mitotic index showed similar results to those previously obtained in the pre- and posttreatment experiments, that is, a lower mitotic index in BLM alone–exposed cells in comparison with control cultures, and no significant differences between combined treatments and BLM-treated cells (data not shown).

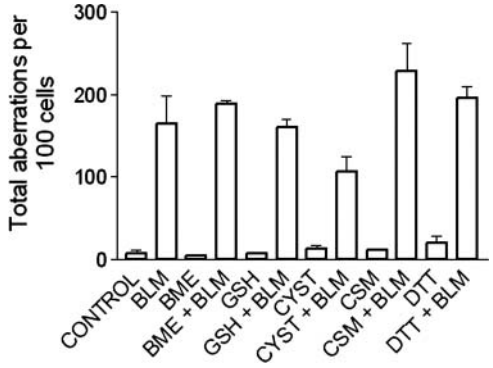

Fig. 7. Effect of thiol compounds on BLM-induced chromosome damage in the G2 phase of cell cycle (30 μg/mL during last 5 hours of cell culture at 37°C) in T-37 cells. Thiols: 10 mM, except CSM and DTT = 0.5 mM, 30 minutes before BLM treatment. Harvesting time: immediately after treatments. Data represent average values of 2 independent experiments (mean ± *SEM*); 100 cells per sample per experiment analyzed.

DNA damage and repair analyzed by neutral and alkaline gel electrophoresis

DNA degradation produces a downward displacement of the DNA smear of the BLM-treated samples, compared with the origin of the smear of the untreated control DNA samples. A modification in the origin of the smear in thiol + BLM combined-treated samples will indicate a protection or

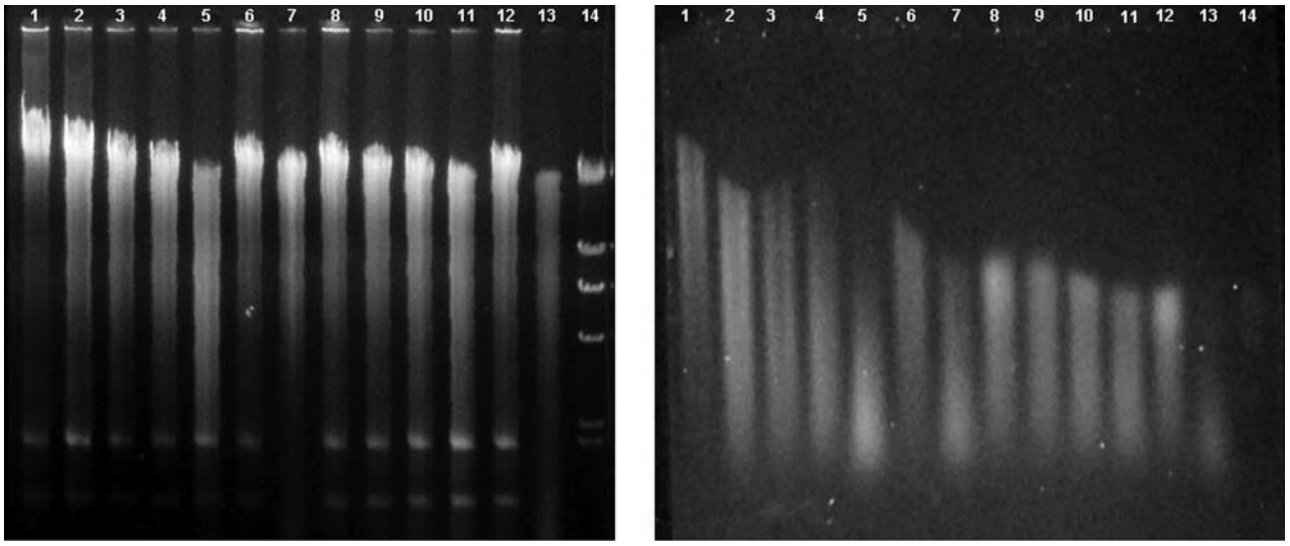

Fig. 8. Photographs of neutral (DSBs) (left) and alkaline (SSBs) (right) electrophoresis gels of DNA damage induced by BLM (100 μg/mL, 37°C). Downward displacements of the origins of smears respect to control denote DNA damage. 1, control (no BLM, no thiols); 2, BLM alone (15 minutes); 3–7, GSH, BME, DTT, CYST, and CSM, respectively (30-minute treatments with 10 mM each) before addition of BLM; 8, BLM alone (45 minutes); 9–13, GSH, BME, DTT, CYST, and CSM, respectively (30-minute treatments with 10 mM each) after addition of BLM; 14, molecular weight marker (lambda/Hind III DNA).

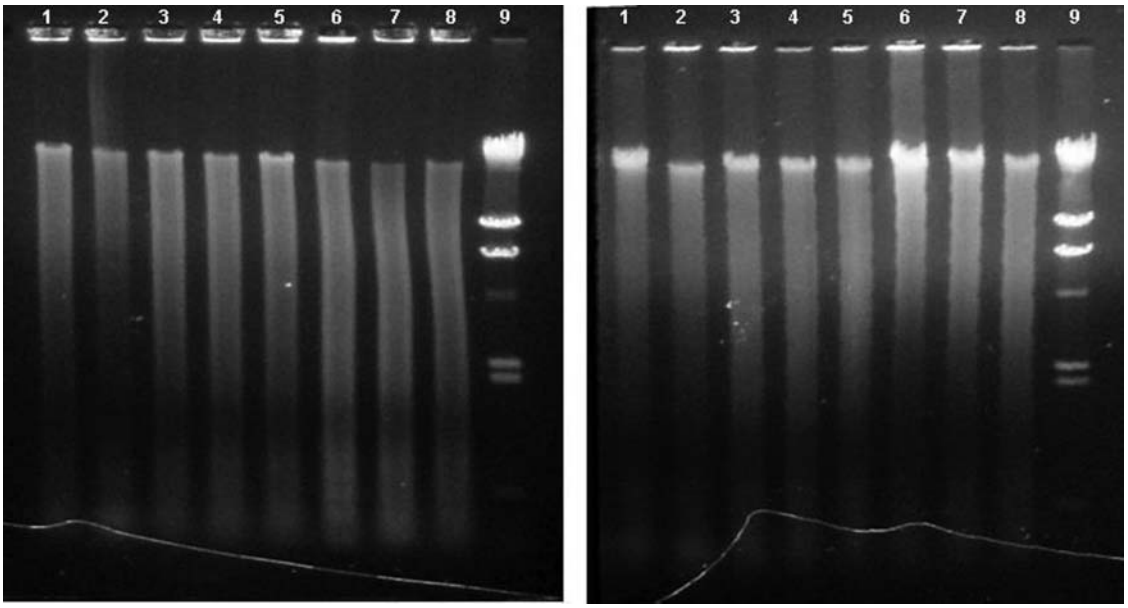

Fig. 9. Photographs of neutral (DSBs) electrophoresis gels of 24-hour repair of DNA damage induced by BLM (100 μg/mL, 37°C). Left photograph: Downward displacements of the origins of smears respect to control denote DNA damage. 1, control (no BLM, no thiols); 2, BLM alone (2 hours, no repair time. DNA was extracted right after BLM treatment); 3, 24-hour repair of BLM (no thiols added); 4–8, 24-hour repair of DNA damage induced by BLM with the previous addition of GSH, BME, DTT, CYST, and CSM, respectively (30-minute treatments with 10 mM each); 9, molecular weight marker (lambda Hind III DNA). Right photograph: Posttreatment with the same thiols. 1, control (no BLM, no thiols); 2, BLM alone (2 hours, no repair time; DNA was extracted right after BLM treatment); 3, 24-hour repair of BLM (no thiols added); 4–8, 24-hour repair of DNA damage induced by BLM after the addition of GSH, BME, DTT, CYST, and CSM, respectively (30-minute treatments with 10 mM each); 9, molecular weight marker (lambda Hind III DNA).

a potentiation by thiols with respect to samples treated with BLM alone. Moreover, if the DNA nicks are repaired, the upper margin of the smears is shifted upwards to a position close to, or even similar to, that of control samples.

Cells were treated with 100 μg/mL BLM and the total DNA was run in a neutral electrophoresis gel to detect double-strand breaks, as shown in Figure 8 (left). BLM produced a marked DNA degradation, as indicated by the downward displacement of the smear with respect to control sample. All samples pretreated with thiols showed more degradation than that of treated with BLM alone, with DTT and CSM showing the highest downward displacement of the smears. When thiols were added to samples with no BLM treatment, no degradation was observed (data not shown). Thiols posttreatments showed a slighter enhancement of DNA degradation than those of pretreated samples. However, once again DTT and CSM showed the highest enhancement of BLM damage and CYST seemed to produce no effect at all (Figure 8, left).

Figure 8 (left) shows a ladder of bands of low molecular weight at the bottom of all lanes. These bands have been reported to result from BLM selected damage in internucleosomal DNA, with the formation of families of fragments corresponding in size to monomers, dimmers, and multimers of the DNA wrapped in the nucleosomes.[5] They can also be the consequence of ongoing cellular necrosis and apoptosis. It is known that this latter process renders 200-bp fragments by nicking of nucleosomes.[33]

A much more remarkable downward of BLM-treated samples was noticed in alkaline gels (Figure 8, right). These gels also showed a greater enhancement of BLM damage by thiol pretreatments. As in neutral gels, DTT and CSM showed the highest potency in potentiating BLM damage. Thiol posttreatment showed similar results than those of pretreatments, but with a slighter effect (Figure 8, right).

Chromosomal aberrations are the consequence of misjoined double-strand DNA breaks. As cytogenetic analysis was performed 24 hours after BLM treatment, we carried out repair experiments of such lesions after 24 hours of cell recovery. The aim of these experiments was to distinguish between DNA repair and any possible protective effects of thiols on the chromosomal aberrations induced by BLM. Cells were treated as for DNA damage experiments, washed, and allowed to mend the damage for 24 hours. Neutral gels showed an enhancement of DNA repair for pretreatments with GSH and BME, whereas all posttreatments showed lesser damage, both compared with BLM alone (Figure 9, left and right).

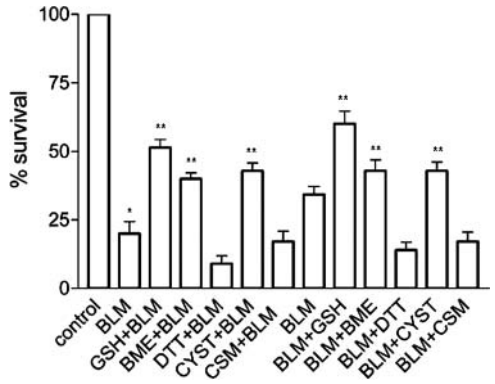

Fig. 10. Effect of thiol compounds on BLM-induced cell killing (100 μg/mL, 2 hours, 37°C) in T-37 cells. Thiols: 10 mM, during 30 minutes before (indicated as respective thiol + BLM) or after (indicated as BLM + respective thiol) BLM treatment. Cell scoring was performed 24 hours after washing thiols and BLM. Data represent average values of 3 independent experiments (mean ± *SEM*). *$p < .01$ compared with control. **$p < .01$ compared with BLM alone (increased survival).

Analyses of cell survival

BLM, at 100 and 500 μg/mL, both after 15 and 45 minutes of pulse treatments, was cytotoxic, and cell survival decreased to values lower than 15% compared with control samples (data not shown). Pre- and posttreatment with all thiols show no significant differences with respect to those obtained with BLM alone. When cells were pulse-treated with BLM 100 μg/mL for 2 hours (reproducing treatments used for cytogenetic experiments), and cell survival scored at 24 hours, GSH, BME and CYST, both in pre- and posttreatments, improved survival by around 20% to 30%, whereas DTT and CSM increased BLM cytotoxicity ($p < .01$) (Figure 10). As this dose of BLM was demonstrated to be cytotoxic even at 24 hours, we determined the LC_{50} dose, which was close to 8 μg/mL. In these conditions, pretreatments with GSH, BME, and CYST increased cell viability by 17%, 14%, and 13%, respectively ($p < .01$). DTT was shown to decrease viability to 17%, and CSM to 11% compared with the LC_{50} alone ($p < .01$) (Figure 11). Posttreatment with GSH, BME and CYST improved survival about 20% compared with the LC_{50} alone, whereas DTT and CSM decreased it ($p < .01$) (Figure 11).

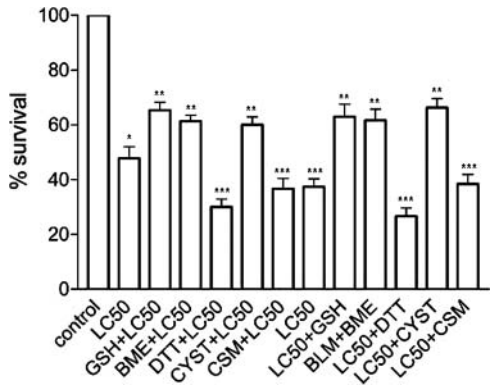

Fig. 11. Effect of thiol compounds on BLM-induced cell survival (LC_{50} = 8 μg/mL, 30 minutes, 37°C) in T-37 cells. Thiols: 10 mM, during 30 minutes before (indicated as respective thiol + LC_{50}) or after (indicated as LC_{50} + respective thiol) BLM treatment. Cell scoring was performed 24 hours after washing thiols and BLM. Data represent average values of 3 independent experiments (mean ± *SEM*). *$p < .01$ compared with control. **$p < .01$ compared with BLM alone (increased survival). ***$p < .01$ compared with BLM alone (decreased viability).

COMMENT

The results obtained in this paper indicate that, when added before BLM, some thiols (ie, BME, GSH, and CYST) can inhibit the clastogenic effect of this antibiotic on human lymphoblastoid cells, and that this inhibitory effect is not due to postreplicative repair induced by these thiols (none of the thiols had a significant effect on the yield of chromatid-type aberrations induced by BLM in the G2 stage of cell cycle). Therefore, we can ruled out homologous recombination (HR), which occurs mainly in S and G2 phases of the cell cycle[34] as a pathway of repair of those double-strand breaks (DSBs) produced by BLM in T-37 cells. The increased DNA repair by BME and CYST is not clear, but it has been reported that the extracellular presence of these thiols increase the level of intracellular GSH.[23,35–40] It has been previously shown that GSH affects DNA DSB rejoining and exchanges.[41–46] The failure in restitution and mis-rejoining of BLM-induced DSBs in the absence of endogenous GSH indicated the involvement of GSH either directly or indirectly in the joining of such DNA DSBs.[46] It is then reasonable to assume that the pretreatment of T-37 cells with BME and CYST could increase GSH, explaining the protective effect of these thiols against BLM-induced clastogenic effects. The lack of a protective effect by thiols when added after BLM treatment indicates that, once BLM reaches the DNA molecule and damages it, thiols are unable to neutralize the genotoxic effect of this compound.

No differences in the MI or in the RI were observed among BLM alone and BLM plus thiols treatments in any of the experiments, which indicates that thiol compounds—at the concentrations used in the present work—did not influence mitotic activity and cell proliferation of BLM-exposed surviving cells.

At the DNA level, all treatments with thiols (before and after BLM)—especially DTT and CSM—increased both single-strand breaks (SSBs) and DSBs induced by BLM. The peak of SSBs and DSBs produced by BLM in T-37 cells was observed at 45 minutes, and the potentiation by thiol posttreatments increased proportionally. The

potentiation of BLM action on DNA by thiols observed in the present study might be the consequence of different mechanisms, acting alone or in combination. As thiols, these compounds may provide electrons for the activation of Fe^{2+}-BLM or for the regeneration of Fe^{2+}-BLM from inactive Fe^{3+}-BLM.[12,29] This might explain the potentiation by all thiols employed in the present study. In addition, CSM also possesses an amine group, and potentiated BLM action on T-37 cells more than GSH, BME, and CYST did. As an amine, CSM binds to DNA[12,28–30] and may alter DNA conformation so as to facilitate BLM action. Widening of the minor groove of DNA, which is the likely site of BLM binding[13,15,47–49] and the location of the 4' position of deoxyribose, may give BLM better access to its target. Rather than being alternatives, the thiol and amine functions may also act in concert, such that the amine groups may bring the thiol group to its site of action on DNA.[12] Although primary, the thiol function is not sufficient to explain the strong potentiation of BLM-induced DNA damage by CSM. Rather, the electrostatic association of this cationic thiol with DNA, mediated by its amino group, concentrates the thiol group at the site of its action on the complex of BLM with DNA. Studies on the plasmid pUC19 using the radiomimetic antibiotic calcheamycin showed that positive thiols have better access to DNA minor groove than neutral or negative ones. Whereas CSM produced chemical repair of deoxyribose moieties, neutral BME, GSH ethyl ester, methyl thioglycolate, and DTT reduced an intermediate radical and a subsequent shifting from a strand breaks to an abasic site. The negative thiols GSH and thioglycolate did not produce any effects.[11] These results suggest that the accessibility of thiols to the site of damage in the DNA is governed by electrostatic interactions between the negative environment of the minor groove and the electric net charge of thiols.

Our data showed that BLM 100 and 500 μg/mL were toxic for T-37 cells, and at 96 hours less than 15% of the cells survived. Therefore, to assess the effect of thiols on cell survival, we determined the LC_{50} to be used in the combined treatments. The better survival produced by these thiols when used in combination with the LC_{50} could be explained by DNA repair. Other processes such as chemical repair of the deoxyribose moieties and scavenging of free radicals in the close proximity of the DNA could also explain the better survival achieved by the cells in the presence of these thiols. Chemical repair did not appear to occur, or at least reactivation of BLM complex by thiols seemed to be the predominant effect. On the other hand, it is not reasonable to assume that GSH could access the deoxyribose inside the DNA due to the negative nature of the minor groove. Scavenging of free radicals by these thiols can explain both less oxidative stress and an inhibition of apoptosis[46] leading to a better survival. GSH may also act as a cofactor of superoxide dismutase so that increasing the scavenging of free radicals by this enzyme.[50–51]

The experiments of cell survival showed that at 24 hours, although GSH, BME and CYST diminished cell lethality compared with cultures pretreated with BLM (100 μg/mL, 2 hours) alone, they increased cell killing by 50%, 40%, and 42%, respectively, when compared with control (no BLM, no thiols) cells. Posttreatments with thiols showed similar results. These results support the hypothesis that DSBs showed by gel electrophoresis correspond to a mixture of DNAs from living and death cells. Moreover, the analyzed DNA can be from apoptotic, necrotic, or intact cells. Therefore, DSBs observed in gels can be a mixture of fragments showing a downward displacement of the smears due to DSBs produced by BLM and those produced by the apoptotic and necrotic processes. On the contrary, metaphase cells analyzed for chromosome damage derived from surviving cells. Thus, the level of chromosome damage estimated through the cytogenetic analysis is an underestimation of the real damage induced at the DNA level.

Thiols lacking an amino group also potentiated BLM genotoxicity in T-37 cells. The thiols CSM and BME are structural analogs, differing only with respect to the amino group. The weakness of BLM potentiation by BME relative to CSM demonstrates the importance of the amino group in the potentiation of BLM-induced DNA damage by aminothiols. The thiol compound CYST, which lacks the net positive charge of CSM, also potentiated BLM genotoxicity in T-37 cells to a lesser degree. These latter findings are in agreement with previous studies.[12] DTT, which is a dithiol, produced the greatest potentiation of DSBs by BLM in T-37 cells. Although its net charge is zero, the presence of 2 sulphydryl groups might determine that it potentiates BLM genotoxicity the most. However, DTT produced dramatic cell lethality even at 24 hours, so the increase in DSBs can be a consequence of apoptosis and necrosis. The order of BLM-induced DNA damage potentiation was negative thiols < neutral thiols < positive thiols, due most probably to electrostatic repulsion between thiols and the negative charge of the DNA.

Previous reports showed a protective effect of GSH,[20] BME,[20] CYST,[21] and CSM[22] pretreatments. Other studies showed potentiation of the BLM action by GSH,[19,25,26] BME,[12,27] CYST,[28] DTT, and CSM.[12,29,30] The discrepancies about thiol effect on BLM action might be due to the use of different experimental conditions and endpoints, and cell cultures of different sources.[12]

The discrepancies observed at cytogenetic (chromosomal aberrations) and molecular (DNA damage) levels by treatments with GSH, BME, and CYST might be explained by DNA repair occurring during the last 24 hours after BLM treatment in cell cultures analyzed for chromosome damage. DNA damage induced by BLM was analyzed immediately after treatments, either by 15 or 45 minutes of incubations with the antibiotic, whereas chromosomal aberrations were scored 24 hours after treatments. Therefore, cells scored for aberrations had more time for DNA repair than cells analyzed for DNA damage. Taken together, cytogenetic and molecular data suggest that the inhibitory effect of thiols on BLM genotoxic effects is not immediate and not due to DNA repair by HR.

To confirm our present results, we are conducting similar experiments using the comet assay to analyze the DNA damage in single cells. These experiments and the ones conducted in this study can help to better understand the significance of the interaction between thiols and BLM, an information of clinical interest because this antibiotic is routinely employed for the treatment of several malignancies.

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For comments and further information, address correspondence to Daniel M. López-Larraza, Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE), C.C. 403, 1900 La Plata, Argentina.

E-mail address: lopezlarraza@ciudad.com.ar

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