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Review

DNA and chromosome damage induced by bleomycin in mammalian cells: An update

Alejandro D. Bolzán[a,b,*], Martha S. Bianchi[a]

[a] Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CICPBA-UNLP-CONICET La Plata), calle 526 y Camino General Belgrano, B1906APO La Plata, Buenos Aires, Argentina

[b] Universidad Nacional de La Plata, Facultad de Ciencias Naturales y Museo, calle 60 y 122, La Plata, Buenos Aires, Argentina

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ABSTRACT

Bleomycin (BLM) is an antibiotic isolated from *Streptomyces verticillus*. It has radiomimetic actions on DNA thus it has been widely used in clinical chemotherapy for the treatment of different types of cancer, including head and neck tumors, lymphomas, squamous-cell carcinomas and germ-cell tumors. Because of this, the study of BLM genotoxicity is of practical interest. This antibiotic is an S-independent clastogen and an agent that generates free radicals and induces single- and double-strand breaks in DNA. In the present review, we will summarize our current knowledge concerning the DNA and chromosome damage induced by BLM in mammalian cells, with emphasis on new developments published since 1991.

1. Introduction

Bleomycin (BLM, CAS No. 9041-93-4) is a radiomimetic antitumor antibiotic first isolated from *Streptomyces verticillus* [1–3]. Actually, this drug belongs to bleomycins (BLMs), a family of glycopeptide-derived antibiotics, which includes bleomycinic acid, BLM A2 and BLM B2, among others [4,5]. BLMs are structurally and biosynthetically related to phleomycins and tallysomycins [5].

BLM is widely used in clinical chemotherapy for the treatment of different types of cancer, namely testicular cancer, lymphoma, lung cancer, cervical cancer and cancers of the head and neck [4,5]. Its clinical formulation Blenoxane is a mixture of components, primarily bleomycin A2 and B2 [2,4,5]. Unlike most anticancer drugs, BLM does not cause myelosuppression, but early development of drug resistance and cumulative lung fibrosis are the major limitations of its use in chemotherapy [6,7]. BLM resistance seems to be associated with reduced DNA damage after BLM exposure, resulting in reduced G2/M arrest and reduced apoptosis [7].

The toxic effects of BLM are thought to be related to its ability to mediate both single-stranded and double-stranded DNA damage, which requires the presence of specific cofactors (a reduced transition metal (Fe(II) or Cu(I)), oxygen and a one-electron reductant) to generate what is called "activated" BLM [4,5]. This chemical species can destroy itself, oxidize lipids, hydrolyze amide bonds of proteins or initiate cleavage events on RNA and DNA molecules – in the latter case through the production of free radicals by activated BLM bound to DNA – that react rapidly and non-specifically with any molecule they encounter [4]. Moreover, BLM can be metabolically inactivated in normal and tumor tissues by an enzyme called BLM hydrolase [5].

Because of the wide use of BLM for the treatment of cancers, the study of its genotoxicity in mammalian cells is of practical interest. As we will see in the next sections, this antibiotic tests positive in the great majority of genotoxicity assays in mammalian cells, including chromosomal aberrations (CAs), micronucleus (MN) and comet assay. In the present review, we will summarize our current knowledge concerning the DNA damaging and clastogenic effects of BLM on mammalian cells, with emphasis on new developments reported in the last 26 years, since the last general review on the genotoxicity of BLM was published by Povirk and Finley Austin in 1991 [2].

Abbreviations: BLM, bleomycin; CAs, chromosomal aberrations; MN, micronucleus; FISH, fluorescent *in situ* hybridization; mtDNA, mitochondrial DNA; SCEs, sister-chromatid exchanges; ITSs, interstitial telomeric sequences; γ-H2AX, phosphorylated histone H2AX; PNA, peptide nucleic acid; CHO, Chinese hamster ovary cells; CHE, Chinese hamster embryo cells; ICE, incomplete chromosome elements; TSSs, DNA cleavage transcription start sites; MMR, mismatch repair; GSH, glutathione; BME, β-Mercaptoethanol; CYST, cysteine; CSM, cysteamine; DTT, ditiothreitol

* Corresponding author at: Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CICPBA-UNLP-CONICET La Plata), calle 526 y Camino General Belgrano, B1906APO La Plata, Buenos Aires, Argentina.

E-mail address: abolzan@imbice.gov.ar (A.D. Bolzán).

URL: http://mailto:adbolzan64@gmail.com (A.D. Bolzán).

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2. DNA damage induced by BLM in mammalian cells

2.1. General remarks

For a review of the chemistry of DNA damage induced by BLM we refer the reader to the excellent articles by Chen and Stubbe [4] and Galim et al. [5], published about a decade ago. In this section of the present review, we will focus on the types of DNA damage induced by BLM on mammalian cells, putting emphasis on the latest developments on this subject.

Several studies have shown that BLM induces base damage, single and double-strand breaks and apurinic/apyrimidinic sites in the DNA molecule (see [2–4] for review). The critical lesions in the cytotoxic effect of BLM are DNA double-strand breaks [2–4], and recent evidence by Chen et al. [8] shows that multiple binding modes of a single BLM molecule can lead to DNA double-strand breaks, that this damage can occur using one or two BLM molecules, and that the ratio single-strand:double-strand breaks varies between the different BLMs. Moreover, Liu et al. [9] using mouse embryo fibroblasts with distinct polymerase β expression levels, showed that BLM-induced DNA damage can be repaired through the base excision repair pathway, and that the absence of this enzyme (in polymerase β deficient cells) promotes oxidative DNA/chromosome damage and gene mutation, which contributes to BLM hypersensitivity. Furthermore, Liddle et al. [10] using Chinese hamster ovary cells (i.e., CHO cell line), showed that BLM-induced γ-H2AX foci (the phosphorylated form of the histone H2AX, which occurs in response to DNA double-strand breaks formation) map preferentially to replicating domains in interphase nuclei.

It is well-known that BLM 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 [4]. Several lines of evidence suggest that DNA damage induced by BLM in living cells is modulated by different factors, including chromatin structure [11–13], DNA repair [12–14], BLM hydrolase [15], antioxidant enzymes [16,17] and thiol-containing compounds [18–23]. Moreover, actively transcribed genes are more susceptible to BLM than silenced genes [11] and BLM-induced DNA damage is cell-cycle dependent [24]. In effect, in synchronized Chinese hamster cells, BLM caused 2–3 times fewer DNA double-strand breaks in S-phase cells than in G1 or G2/M phase cells, i.e., during S-phase BLM produces DNA damage, but in a lesser extent than in G1 or G2 phases [24]. This observation is in good agreement with early studies in mammalian cells (HeLa and CHO) showing that BLM does not directly interfere with DNA replication (i.e., it does not inhibit the initiation and completion of DNA synthesis) [25,26].

The more recent findings on the DNA damaging effects of BLM on mammalian cells refer mainly to those ones made using the comet assay to detect DNA strand breaks, and molecular biology techniques to analyze the effect of this compound on telomeric and mitochondrial DNA (mtDNA) and to determine the genome-wide pattern of DNA cleavage by BLM. Next, we will briefly discuss these findings.

2.2. BLM-induced damage on telomeric and mitochondrial mtDNA. Other studies on BLM-induced DNA damage in mammalian cells

More than a decade ago, Arutyunyan et al. [27] applied for the first time the Comet-FISH technique (i.e., single cell gel electrophoresis or comet assay in combination with fluorescent *in situ* hybridization or FISH) with a telomere-specific peptide nucleic acid (PNA) probe, to analyze the damage induced by BLM (and also mytomicin C) on telomeric DNA. These authors found that both anticancer drugs induce fragmentation (breaks) in telomere-associated DNA in human lymphocytes. However, BLM and mytomicin C induced DNA breaks in the DNA of or adjacent to telomeric repeats were found to be proportional to that of total DNA, which suggests random induction of DNA breaks by these two antibiotic compounds. The same year, Milić and Kopjar [28] performed a similar study but using the alkaline comet assay alone and found that both drugs and their combination induce a significant DNA damage, showing a synergetic effect in these cells, although BLM alone induced the highest level of damage. A year later, Arutyunyan et al. [29], using Comet-FISH, also showed that, in human lymphocytes, BLM induces telomere DNA damage. The induction of telomere DNA damage by BLM in mammalian cells was confirmed by Hovhannisyan et al. [30], who analyzed the effect of these drugs in normal human leukocytes and three transformed cell lines (HT1080, CCRF-CEM and CHO) using Comet-FISH. It was shown that telomeres in CHO and CCRF-CEM cells were about 2–3 times more sensitive towards BLM than global DNA, while in HT1080 telomeres were less fragile than total DNA. Moreover, these authors found significant differences between the above cell lines with respect to quantitative head/tail distribution of telomeric signals after BLM exposure: while a large number of telomeric signals of various sizes were found in CHO cells, very small signals were detected in the comets of HT1080 and CCRF-CEM lines. This is probably due to the fact that CHO cells contain large blocks of interstitial telomeric repeats [31], while human tumor cell lines have short telomeres [32]. A further analysis of the studies of DNA damage by BLM and other anticancer drugs using Comet-FISH can be found in the review article of Glei et al. [33]. Recently, Liu et al. [34,35], studied the effect of BLM and other anticancer drugs on telomeres of a mouse spermatogonial cell line and rat male germ cells (from Brown Norway rats). The co-localization of telomere and γ-H2AX signals after FISH and immunofluorescence, respectively, observed in these cells, indicated that BLM damages telomeric DNA of germ cells.

A few years ago, Nguyen and Murray performed a pair of studies to determine the effect of BLM on human telomeric DNA using DNA sequencing [36,37]. These studies showed that human telomeric DNA sequences are a major target for this anticancer drug [36,37]. They examined the DNA sequence specificity of BLM in a target DNA sequence containing 17 repeats of the human telomeric sequence and other primary sites of BLM cleavage and found that BLM cleaved primarily at 5′-GT in the telomeric sequence 5′-GGGTTA [36,37]. The telomeric region constituted 57% of the 30 most intense BLM damage sites in the DNA sequence examined, which indicates that telomeric DNA sequences are a major target for BLM damage. More recently, Chung and Murray [38], using end-labeled DNA and capillary electrophoresis, analyzed the DNA sequence specificity of BLM in two human mtDNA sequences. This compound was found to cleave preferentially at 5′-TGT*A-3 DNA sequences (where * is the cleavage site). Previously, Yeung et al. [39] analyzed the mtDNA damage induced by BLM in acuted myeloid leukemia cells and reported that this compound damaged mtDNA at concentrations that induced cell death.

On the other hand, the alkaline comet assay was also employed to analyze the rejoining kinetics of BLM-induced DNA damage in human lymphocytes [40]. These authors detected early (0–30 min) events in the induction of single-strand breaks in the lymphocytes of 45 individuals, and showed that, after DNA damage induction, the fastest return to the background level occurred in 5 min, whereas the lowest return took approximately 30 min [40]. Besides, the early rejoining kinetics of single-strand breaks showed multiple patterns, depending on the individual analyzed. Moreover, Weng et al. [41], analyzed the DNA damage induced by BLM and H_2O_2 in different subpopulations of human white blood cells using the comet assay and found that they differ in their sensitivity to these compounds, B-cells showing the highest sensitivity to BLM.

2.3. Studies on the genome-wide pattern of DNA cleavage by BLM in human cells

In 2014, Murray and coworkers [42] investigated the genome-wide pattern of DNA cleavage by BLM at transcription start sites (TSSs) of actively transcribed and non-transcribed genes in human HeLa cells, using next-generation DNA sequencing. They found that actively

transcribed genes were preferentially cleaved compared with non-transcribed genes, since the BLM cleavage pattern at highly transcribed gene TSSs was greatly enhanced compared with purified DNA and non-transcribed gene TSSs. The pattern of BLM enhanced cleavage showed peaks that were approximately 200 bp apart, this finding indicating that this compound identified the presence of phased nucleosomes at TSSs. These authors, by using next-generation DNA sequencing, also reported that in HeLa cells the repair of BLM-induced 3′-phosphoglycolate termini was enhanced at actively transcribed genes [43].

Complementing the above studies, Murray et al. [44] determined for the first time, the genome-wide DNA sequence specificity of BLM breakage in human cells. They examined over 200 million BLM cleavage sites using next-generation DNA sequencing techniques. Mostly, the preferred site of BLM breakage was at 5′-GT* dinucleotide sequences, with lesser cleavage at 5′-GC* dinucleotides. These authors also determined the presence of longer BLM cleavage sequences, with preferred cleavage at 5′-GT*A and 5′-TGT* trinucleotide sequences, and 5′-TGT*A tetranucleotides. In the case of cellular DNA, the most highly cleaved DNA sequence was the hexanucleotide DNA sequence 5′-RTGT*AY (where R corresponds to a purine and Y indicates a pyrimidine).

In summary, the most recent studies on BLM-induced DNA damage in mammalian cells confirm that this antibiotic induces DNA fragmentation and show that it damages telomeric and mtDNA. Besides, these studies show that human telomeric DNA is a major target for BLM, and that actively transcribed genes are preferentially cleaved by this compound compared with non-transcribed genes, being 5′-GT* dinucleotide sequences the preferred site of BLM breakage in human cells.

3. Chromosome damage induced by BLM in mammalian cells

3.1. BLM is an S-independent clastogen

Like ionizing radiation, BLM produces free radicals and acts on chromosomes in an S-independent manner, inducing CAs in any period of interphase, presumably as a result of its ability to induce DNA single- and double-strand breaks [2–5]. For the above reasons, BLM is considered a radiomimetic compound. Besides, with rare exceptions, BLM is a poor inducer of sister chromatid exchanges (SCEs), inducing no increase or only a small increase at very high concentrations (see [2] for review). The first studies on the induction of CAs by BLM were made by Ohama and Kadotani in human lymphocytes, as early as in 1970 [45]. This and other studies made in the following decades showed that BLM produces both chromosome- and chromatid-type aberrations in all the systems studied (see [2] for review). The techniques used to determine the chromosome damage induced by BLM included the standard technique of scoring CAs using Giemsa staining, premature chromosome condensation, and the MN test [2]. For a review on the early studies about the chromosomal effects of BLM we refer the reader to the review article by Povirk and Finley Austin [2]. Next, we will focus on the most important findings on this subject published after that review. But before that, it is important to mention two studies referring to the factors that influence the chromosomal sensitivity to BLM. In one of them, Allío and Preston [46] reported that human lymphoblastoid cells deficient in tumor suppressor genes ATM and p53 are more sensitive to BLM (G2-induced chromatid aberrations) than wild-type cells, this finding indicating that the chromosomal sensitivity to BLM results from alterations in the DNA damage response pathway. A few years later, Vernole et al. [47] examined the effects of either mismatch repair(MMR)-deficiency or p53 inactivation, or both, on cellular responses to BLM using the MMR-deficient colon carcinoma cell line HCT116 and its MMR-proficient subline HCT116/3–6, both expressing wild-type p53, transfected with an expression vector encoding a dominant-negative p53 mutant, or with the empty vector. Their data showed that loss of MMR and p53 function exerts opposite and independent effects on chromosome damage induced by BLM, since loss of MMR function alone was associated with increased resistance to chromosome damage by the drug, whereas loss of p53 alone resulted in increased sensitivity to this compound [47].

3.2. BLM-induced chromosome damage as assessed by FISH with chromosome painting probes

In 1993, Hoffmann et al. [48,49] investigated the induction of MN by BLM in human lymphocytes and its potentiation by radioprotectors. A year later, these authors used for the first time, the FISH technique with a chromosome painting probe (for chromosome 4) to analyze the clastogenic effects of BLM in human lymphocytes [50]. They found that the aminothiol radioprotectors 2-[(aminopropyl) amino] ethanethiol (WR-1065) and cysteamine (CSM) potentiate the induction of CAs and MN by BLM in G0 human lymphocytes [49,50]. According to Hoffmann and coworkers, this effect can be explained by the combination of a thiol-mediated redox mechanism and an amine-mediated targeting of the thiol function to DNA [51]. In effect, as thiols, both WR-1065 and CSM may donate electrons for the activation of BLM-Fe^{+2} or the regeneration of activated BLM from inactive BLM. Moreover, the cationic nature of WR-1065 and CSM, conferred by the amino groups, concentrates the active thiol function at the site of BLM action on DNA, thus the diamine thiol WR-1065 is a more effective potentiator of BLM damage than is the monoamine thiol CSM [51].

A year later, Ellard et al. [52] analyzed the chromosome damage induced by BLM in human lymphocytes but using simultaneously three chromosome painting probes (for human chromosomes 1, 2 and 3), and showed that BLM induces complex exchange aberrations (i.e., exchange aberration involving three or more breaks in two or more chromosomes) in these cells. The FISH data indicated a slightly greater proportion of symmetric vs. asymmetric exchanges: The ratio of BLM-induced translocations:dicentrics was 1.15. These authors pointed out that, due to the reduced resolution of chromosome morphology following FISH, it is possible that some dicentrics could have been misclassified as translocations. After this study, several groups reported the use of FISH with chromosome painting probes to analyze the chromosome damage induced by BLM in mammalian cells. In 1996, Wu et al., using cultured lymphocytes of lung cancer patients, reported that BLM induces breaks preferentially on chromosomes 4 and 5 [53]. This study was performed to assess whether the chromatid breaks induced by BLM could survive as chromosome-type aberrations after treated lymphocytes were allowed to recover in a drug-free medium for one or two cell generations, and whether the survival rates of lesions on these chromosomes differed between lung cancer patients and controls. These authors found that, in samples allowed to recover for 48 h, most aberrations were of the chromosome-type [53]. The proportion of chromosome 5 aberrations surviving as chromosome-type aberrations was significantly higher in the cells of lung cancer patients than in controls, thus these authors proposed that chromosome 5 lesions in human lymphocytes could be used as a biomarker to identify populations at risk for lung cancer [53]. A few years later, Puerto et al. [54] by applying FISH with painting probes for chromosomes 1 and 4 to human lymphocytes, found that these chromosomes are equally sensitive to BLM. However, the high gene density chromosome 1 appeared to be more sensitive to repair inhibition by cytosine arabinoside (Ara-C) of BLM-induced damage than chromosome 4, a finding that could be related to preferential repair of open chromatin and actively transcribed regions [54]. The same year, Xiao and Natarajan, using FISH with arm-specific painting probes for Chinese hamster chromosomes 3, 4, 8 and 9 in Chinese hamster primary embryonic cells treated at G1 phase with BLM, reported that the frequency of insertions was approximately equal to that of reciprocal translocations and that the frequency of induced pericentric inversions was higher than that of centric rings [55]. In addition, they found that these chromosomes were differentially involved in the aberrations induced by BLM: taking into account a

random distribution of aberrations on the basis of the relative length of the chromosomes or chromosome arms, chromosome 8 was found to be more involved than expected, chromosome 4 was randomly involved, and chromosomes 3 and 9 were less involved [55]. In 1998, Mosesso et al. [56] analyzed the chromosome damage induced by BLM in human lymphocytes under simulated microgravity conditions using FISH with chromosome painting probes and found that, under these conditions, the levels of dicentrics and rings, the ratio symmetrical:asymmetrical translocations, the number of cells with complex aberrations, and the total numbers of aberrations were significantly elevated compared with parallel treatments performed as 1g control or normal ("ground"). Thus, they concluded that simulated microgravity promotes the misrejoining of double-strand breaks, leading to the fixation of original lesions in the DNA into CAs, and that microgravity favors the production of symmetrical translocations over asymmetrical ones [56]. Thus, microgravity promotes BLM-induced chromosome damage in human lymphocytes. Recently, Lu et. al [57] performed a similar study, but at the DNA level, analyzing the cellular responses and gene expression profile changes induced by BLM in human fibroblasts in space. The study was conducted in the International Space Station using confluent human fibroblasts treated with BLM for three hours in a true microgravity environment, and DNA damage was evaluated by γ-H2AX foci and microarray analysis. Although similar damage was found between flight and ground cells in terms of γ-H2AX foci (though there was a slight shift in the distribution of foci counts in the flown cells, probably due to differences in the proliferation rate between flight and ground cells), microarray analysis showed that some genes involved in DNA damaging signaling were significantly upregulated in both flight and ground cells after BLM treatment [57]. Thus, these authors concluded that whether microgravity affects DNA damage response in space can be dependent on the cell type and cell growth condition.

Very recently, using FISH in combination with the MN test, Hovhannisyan et al. [58], characterized the chromosome content of MN in human lymphocytes treated with BLM and mitomycin C. They used centromeric and whole-chromosome painting probes (for chromosomes 1, 9 and 16 in the case of BLM) and found that the MN contained material derived from all chromosomes investigated, and that the frequencies of involvement of the above chromosomes correlated with the size of the MN. Additional studies using cytogenetic techniques to determine the clastogenic effects of BLM were also performed, but within the framework of adaptive response studies and the BLM sensitivity assay, and thus will be considered in the corresponding sections of the present review (see below, Sections 4 and 5).

In recent years, the use of FISH with telomeric probes allowed to determine the effects of BLM on the telomeres and interstitial telomeric sequences (ITSs) of mammalian chromosomes, as we will see in the next sections of this review.

3.3. Clastogenic effects of BLM on mammalian telomeres

In an early study, we investigated the involvement of the vertebrate telomeric repeat sequence (TTAGGG)n in the CAs induced by the radiomimetic compounds BLM and streptonigrin in Chinese hamster cells (CHO and CHE cell lines) [31]. To this end, we used a PNA telomeric probe which, because of its neutral backbone, penetrates into the chromosome rather than only bind to the surface of it, thus providing a high efficiency in the detection of (TTAGGG)n repeats [59]. Since this probe does not recognize subtelomeric sequences, it is highly specific and the fluorescence intensity of the spots is directly correlated to the length of the telomeres [59]. FISH with the PNA telomeric probe showed that 18% of the scored aberrations induced by BLM in CHO cells exhibited telomeric repeat signals, whereas 29% of the total aberrations induced by BLM in CHE cells involved telomeric repeat sequences [31]. The localization of the telomeric FISH signal depended on the type of aberration analyzed. In dicentrics and rings, the telomeric signals were observed at the centromeric regions of chromosomes; in triradials and quadriradials the telomeric signal was located at the rearrangement site, whereas in the acentric fragments, the telomeric signals were present at one or both ends. Besides, acentric fragments labeled along their entire length and translocations of telomeric repeat sequences (detected as chromosomes with several interstitial telomeric signals) were also found in both cell lines [31]. Since the observed frequencies of chromosomal aberrations involving telomeric repeats induced by BLM and streptonigrin in both cell lines were higher than expected according to the percentage of the genome labeled with the telomeric probe, we concluded that telomeric repeat sequences are preferentially involved in chromosome breakage, fragility and recombination induced by radiomimetic agents in mammalian cells. In addition, some of the damaged CHE cells exhibited one or more chromosomes with additional zones of hybridization, indicating the possible amplification of (TTAGGG)n repeats, a phenomenon not related to telomerase activity [31].

A few years later, a more detailed study was carried out in order to analyze the clastogenic effect of BLM on mammalian telomeres [60]. By using FISH with a PNA telomeric probe, we found that BLM induces chromosome elements with telomeric signal at only one terminal end (the so-called "incomplete chromosome elements" or ICE, which includes incomplete chromosomes – chromosomes with one or more centromeres and lacking one or both ends – and terminal fragments) [61], and interstitial fragments (acentric fragments lacking both ends) in Chinese hamster embryo cells (CHE cell line). In other words, BLM induces telomere instability in the form of chromosome end loss in mammalian cells.

At this point, it is important to remember that telomere instability refers to the chromosomal instability caused either by the loss of the chromosome ends (one or both) or the dysfunction of telomeres [61]. These phenomena can take place in the short (at first cell división after the induction of chromosome damage by a given mutagen) or in the long term (in the progeny of the exposed cells). Once telomere instability arises, the involved chromosomes tend to associate or fuse with each other [61]. Besides the above findings, interstitial fragments and ICE were found to be the most frequent asymmetrical chromosomal aberrations induced by BLM in CHE cells [60]. In order to confirm previous observations, in 2006 we analyzed the induction of ICE by BLM in two mammalian cell lines, the abovementioned CHE cell line (average 2n = 23) and the CPC cell line (from domestic rabbit embryo skin fibroblasts, average 2n = 44) [62]. BLM induced ICE, dicentrics, and interstitial acentric fragments in CHE cells, but only ICE in CPC cells. Almost 100% of the BLM-induced ICE in both cell lines consisted of pairs formed by an incomplete chromosome and a terminal fragment [62]. These results confirmed that ICE are the most frequent type of unstable chromosomal aberration induced by BLM in mammalian cells.

In 2008, Benkhaled et al. [63] investigated the induction of ICE by BLM in human lymphocytes by applying fluorescence plus Giemsa (FPG) and FISH using pan-centromeric and pan-telomeric probes. Telomere and centromere FISH allowed to determine that the ratio between total incomplete elements and multicentrics was 0.27 (similar to the one induced by low-LET radiation) [63]. This contrasts with our previous findings in CHE cells [62], in which a ratio of ICE/dicentrics of 27.6 for BLM-exposed cells was found. The different sensitivity to BLM between the CHE cells and human lymphocytes could be attributed to different factors [63] and suggests that the effect of clastogenic agents observed in cell lines cannot be directly extrapolated to human lymphocytes. Moreover, Benkhaled et al. [63] confirmed our previous observations in CHE cells, indicating an elevated proportion of interstitial fragments in relation to total acentric fragments in human lymphocytes treated with BLM, which could be a characteristic signature of the clastogenic effect of this compound on mammalian cells.

Finally, Paviolo et al. [64] analyzed the long-term effect of BLM on the telomeres of rat cells (the ADIPO-P2 cell line, derived from adipose cells from Sprague-Dawley rats) by using also FISH with a PNA telomeric probe, and found that BLM induces persistent telomere instability

in mammalian cells, cytogenetically manifested as incomplete chromosome elements (i.e., chromosome end loss) and telomere FISH signal loss and duplication (i.e., telomere dysfunction) [64]. This instability persists for several generations after exposure (at least for 10 days after treatment). Moreover, the appearance of telomere fusions in BLM-exposed cells 10 days after treatment suggests that this compound can induce delayed telomere instability. The delayed appearance of dicentric chromosomes and telomere fusions (which produces dicentric chromosomes without accompanying fragment) that we observed in ADIPO-P2 cells exposed to BLM suggests that the so-called breakage-fusion-bridge (BFB) cycles [65] might play a significant role in the maintenance of the long-term telomere instability induced by this compound. Thus, by inducing breakage at terminal regions of chromosomes, resulting in incomplete chromosomes, BLM could promote genome instability through BFB cycles, which can continue for multiple cell generations, leading to extensive chromosomal rearrangements in the progeny of the cells exposed to this compound. Moreover, the persistent telomere instability induced by BLM found in rat cells was neither related to telomerase activity [64] nor telomere length variations [Paviolo et al., unpublished].

3.4. Effects of BLM on mammalian interstitial telomeric sequences (ITSs)

ITSs are those blocks of telomeric repeats [(TTAGGG)n repeats in mammals and other vertebrates] present in non-terminal regions of chromosomes, and include those intrachromosomal telomeric-like repeats located near (pericentromeric ITSs) or within the centromere (centromeric ITSs) and those telomeric repeats located between the centromere and the telomere (i.e., truly interstitial telomeric sequences) of eukaryotic chromosomes [66]. Although the involvement of ITSs in the chromosomal aberrations induced by BLM had been previously investigated in CHO cells using telomere FISH, in 2009 we decided to perform a more detailed study [67] to determine the effect of this compound on mammalian ITSs. CHO cells were exposed to increasing concentrations of BLM and chromosomal aberrations were analyzed in the first mitosis after treatment. We found that most of the chromosome breaks induced by BLM exhibiting telomeric signals occurred in the centromeric regions of chromosomes. This observation, along with the finding of entirely labeled acentric fragments in BLM-exposed cells but not in untreated cells, showed that this antibiotic induces breakage at chromosomal sites containing ITSs. However, since the percentage of entirely labeled acentric fragments induced by BLM was lower than expected taking into account the percentage of the genome occupied by telomeric repeats and the percentage of telomeric signals represented by the centromeric ones, we concluded that centromeric regions containing ITSs are not the preferential target for BLM clastogenic action. In addition, our results showed that heterochromatic ITSs are involved more than expected in the formation of chromosome/chromatid breaks induced by BLM, taking into account the percentage of the genome covered by telomeric sequences [67]. Moreover, our results show that BLM is capable of inducing amplification and translocation of telomeric repeats. On the other hand, our results showed that BLM treatment increases the size of ITSs and that this effect is not related to the chromosomal sensitivity of the exposed cells to this compound [67].

More recently, we analyzed the long-term effect of BLM on mammalian ITSs in the progeny of CHO cells exposed to this compound, in order to determine if these telomeric-like sequences play some role in the long-term clastogenic effect of this antibiotic [68]. We found that BLM induces ITSs instability, cytogenetically detectable as acentric fragments (18 h after treatment) or additional (new) FISH signals (6 days after treatment) [68]. We proposed that the delayed effect of BLM on ITSs mainly results from breakage of heterochromatic ITSs blocks and further insertion of these sequences at the sites of monochromatid breaks occurring at G2 phase of the cell cycle, since most of the additional FISH signals were present as single dots and located at interstitial sites of the involved chromosomes [68].

In summary, recent studies on the clastogenic effects of BLM on mammalian cells show that this compound induces complex CAs (involving three or more breaks in two or more chromosomes), ICE (which implies breakage events at the chromosome ends) and other telomeric-related aberrations (such as telomere duplication or fusion) which persist several generations after treatment, and damages ITSs, producing breakage, amplification and translocation of these sequences. In addition, a few reports [53,55] suggest that the chromosomal sensitivity to BLM in mammalian cells varies between chromosomes, although further studies are needed to confirm this assumption.

4. Use of BLM to detect an adaptive response to mutagens in mammalian cells. Adaptive response to BLM-induced genotoxic effects in mammalian cells

The adaptive response is a phenomenon observed in cells exposed to low, non-genotoxic doses of a mutagen after a challenge treatment with the same or another mutagen. Thus, after the challenge treatment, these cells become less susceptible to mutagen-induced cytogenetic damage. In the last decades, several studies were carried out in order to determine if human cells exposed to ionizing radiation exhibit an adaptive response able to be observed after a challenge treatment with BLM. Some of them employed CAs as endpoint to determine the adaptive response, whereas others used DNA damage (assessed by the comet assay) as endpoint. In 1995, Tedeschi et al. [69] demonstrated that cultured lymphocytes from children, still contaminated by the fallout of Chernobyl accident (which happened on April 26th, 1986), exhibit resistance to the clastogenic action of BLM challenge treatment. A year later, the same authors analyzed the possible adaptive response, induced in vivo by exposure to ionizing radiation to a challenge treatment with BLM in lymphocytes from children living in the town of Pripjat (situated 3 km NW of the nuclear plant) at the time of the Chernobyl accident (so they were exposed to the acute initial high dose of ionizing radiation) [70]. Significantly lower chromosome damage (BLM-induced chromatid aberrations in G2) was found only in lymphocytes from children who, independently of the initial acute exposure to ionizing radiation, still showed a ^{137}Cs (radiocesium) internal contamination, due to persistent continuous exposure to low doses of radiation. These authors conclude that past exposure to acute high dose of ionizing radiation did not induce or affect resistance to BLM in these children, i.e., hyposensitivity or resistance to BLM is induced by internal contamination, due to the continuous exposure to low doses, rather than by the hit of a past acute exposure to relatively high doses of ionizing radiation [70]. The same year, Barquinero et al. investigated a group of 12 individuals occupationally exposed to ionizing radiation and 11 unexposed ones to determine if a challenge dose of BLM could induce an adaptive response on their lymphocytes [71]. They found that, after BLM treatment, the frequencies of chromatid-type aberrations (breaks and gaps) were significantly lower in exposed individuals than in the unexposed ones, this finding suggesting that occupational exposure to ionizing radiation can induce an adaptive response, detectable by a subsequent treatment with BLM [71]. In 2001, Frenzilli et al. [72] analyzed if an adaptive response could be observed after a challenge treatment with BLM (evaluated by the comet assay) in children of Chernobyl 10 years after the disaster. They evaluated the extent of DNA damage in the lymphocytes of 43 Belarussian children (16 healthy and 27 affected by thyroid cancer) and thirty-nine healthy children from Pisa (Italy) enrolled as controls. These authors found no differences in the sensitivity of lymphocytes from different groups of children to BLM, this finding indicating the absence of an adaptive response [72]. However, since the design of the study was wrong (i.e., one group included healthy children and children with cancer, whereas the control group consisted of healthy children from other location), this conclusion should be taken with caution.

Finally, in 2015, Zong et al. [73] analyzed if radiofrequency fields

(900 MHz) were able to induce an adaptive response able to be detected after a challenge treatment with BLM and found that mice exposed to both agents showed a significantly reduced BLM-induced DNA damage. This finding suggests that radiofrequency exposure is capable of inducing an adaptive response in the lymphocytes of mice able to be revealed with a challenge treatment with BLM.

On the other hand, two studies analyzed specifically the adaptive response to BLM. In one of them, Schlade-Bartusiak [74] analyzed the adaptive response to BLM and mitomycin C in human lymphocytes using CAs for BLM and SCEs for mitomycin C as endpoints. They found interindividual variations in the chromosomal sensitivity to these compounds, and that the pre-treatment with a low dose of BLM produced almost a 50% decrease in the frequency of aberrations induced by a challenging dose, while the protective effect of mitomycin C was less than 20% [74]. These authors related the higher adaptive response induced by BLM to the repair processing of the induced DNA damage. In the other study, Krishnaja et al. [75] analyzed the variability in cytogenetic adaptive response of cultured human lymphocytes to different mutagens, including BLM, using CAs, SCEs and MN as cytogenetic endpoints. Adaptation to BLM and hyperthermia showed cross-resistance to chromosome damage induction by gamma rays and BLM/mitomycin C, respectively. Cell cycle analysis indicated that adaptation to BLM is not caused by a change in the rate of cell proliferation after challenge dose.

In summary, while BLM has been proven to be useful to detect an adaptive response to ionizing radiation or radiofrequency fields, further studies are needed to confirm the induction of an adaptive response by BLM in mammalian cells and the factors that influences it.

5. The BLM sensitivity test: evaluation of the DNA repair capacity of human cells

In the last two decades, BLM has been used as one of the main compounds to test the DNA repair capacity of cells or individuals through the so-called “mutagen sensitivity assay” or “mutagen sensitivity test”. In this assay, mutagen sensitivity is determined in peripheral blood lymphocytes as the mean number of chromatid breaks per cell (b/c) at metaphase induced *in vitro* by BLM exposure in the late S-G2 phase of the cell cycle [76]. Cells of persons deficient in DNA repair respond with high numbers of BLM-induced chromatid breaks. Therefore, a high number of BLM-induced b/c is strongly associated with the development of environmentally related cancers, such as colon cancer, lung cancer, and head and neck squamous cell carcinoma (see [76,77] for review). In recent years, besides the classic BLM sensitivity assay using chromatid breaks as endpoint, alternative versions of this assay emerged, using chromosome damage (assessed by the MN test or by FISH with chromosome painting probes), DNA damage (evaluated by the comet assay) or even microarrays as endpoints.

Several studies were performed in the last decades using the different versions of the BLM sensitivity test to evaluate the DNA repair capacity in human cells. In 1999, Cloos et al. [78] analyzed the BLM-induced chromosome damage in human lymphocytes and primary oral fibroblasts and keratinocytes using the classic BLM sensitivity test, and found that oral keratinocytes were extremely sensitive to BLM, and concluded that oral fibroblasts can be used to measure BLM-sensitivity in terms of chromatid breaks. A year later, Roy et al. [79] applied this test to breast cancer families and found that the mean frequency of BLM-induced aberrations per cell was significantly higher among breast cancer patients compared to their healthy blood relatives and control subjects. Lymphocytes from healthy blood relatives also showed increased sensitivity to BLM. The cancer risk calculated for breast cancer patients was fourfold higher than that of controls [79]. Also in 2000, Zych et al. [80] applied the mutagen sensitivity assay but using both classical staining and FISH with chromosome painting probes to analyze BLM-included CAs in head and neck cancer patients, showing the usefulness of FISH as a complementary method to the standard mutagen sensitivity assay to determine the chromosomal sensitivity of human cells to BLM. In 2001, Barquinero et al. [81] reported that G2 lymphocytes of Fanconi Anemia heterozygotes exhibit a high in vitro sensitivity to BLM, an effect not observed with ionizing radiation. In 2002, Cloos et al. [82] investigated whether cell cycle control is involved in the mutagen sensitivity induced by BLM, using 21 lymphoblastoid cell lines with different mutagen sensitivity score, and an ataxia telangiectasia cell line included for comparison. They found that BLM-treated hypersensitive cells remained at a relatively high level of DNA synthesis, (measured by thymidine incorporation), and showed a decreased accumulation of cells in G2 and M phase (measured by flow cytometry) compared to BLM-insensitive cell lines. Ataxia telangiectasia cells were found to be highly sensitive to BLM and exhibited a high level of DNA synthesis and a strong G2 block. Thus, BLM sensitivity seems to be associated with “damage-resistant growth," which is indicative of impaired cell cycle arrest [82]. A year later, Lin et al. [83] analyzed the use of the BLM sensitivity assay as a susceptibility marker for endometriosis and found a significant difference with regard to mean chromatid breaks per cell between women with and without endometriosis, which suggests that chromosomal sensitivity to BLM of peripheral lymphocytes is associated with the risk of endometriosis development. In 2004, Tedeschi et al. [84] assessed the validity of aphidicolin and BLM-induced chromosome damage in cultured human lymphocytes as biomarker of mutagen sensitivity. They evaluated chromosome damage by the analysis of CAs and the MN test in peripheral blood lymphocytes from 9 monozygotic and 10 dizygotic healthy male twins aged 70–78 years to determine whether mutagen sensitivity has a genetic rather than an environmental basis. Differences in response between identical and not identical twins revealed a high genetic component in the sensitivity to aphidicolin (both for CAs and MN), BLM and BLM plus aphidicolin treatments (MN only) [84].

In 2005, Wei et al. [85] evaluated the DNA repair capacity of cancer patients with the BLM challenge test (BLM 20 mg/ml for 30 min and repaired for 15 min) and the UVC challenge test (UVC 254 nm at the dose of 1.5 Jm2 and measured before exposure and at 90 and 240 min after UVC exposure). They used human peripheral lymphocytes collected from 33 patients with different kinds of cancers and 33 controls in the same hospital. Unlike previous studies, Wei and coworkers used the comet assay to evaluate mutagen sensitivity (DNA repair capacity) instead of the classic chromatid-aberrations test. They found that the DNA repair capacity measured with the above challenge tests in cancer patients was significantly lower than that in controls, i.e., these individuals were highly sensitive to BLM and UVC [85]. A year later, Chao et al. [86] conducted a prospective study to determine whether mutagen sensitivity to BLM in peripheral blood lymphocytes was associated with the future development of cancer in patients with Barrett’s esophagus. The found that sensitivity to BLM is associated with an increase of risk of cancer progression in persons with Barrett’s esophagus. Also in 2006, Cloos et al. [87] employed another variant of the mutagen sensitivity test, using microarrays analysis to evaluate the BLM-induced damage in human cells (lymphoblastoid cell lines generated by immortalization of blood lymphocytes with Epstein Barr virus) belonging to individuals with low and high mutagen sensitivity, in order to uncover those genes involved in susceptibility to sporadic cancers. To this end, these authors compared gene expression of BLM-insensitive (with low breaks per cell score after BLM exposure, i.e., 0.60 breaks per cell on average) vs. BLM-sensitive (with high breaks per cell score after BLM treatment, i.e., 1.04 breaks per cell on average) cells. The expression of several genes resulted altered in the sensitive group (46 genes showed higher expression, whereas 55 genes exhibited lower expression) after BLM exposure, including many genes involved in biological processes (such as cell growth and/or maintenance, proliferation, and regulation of cell cycle), as well as some genes involved in DNA repair [87]. These results showed that BLM induces the alteration of cellular processes, resulting in a retardation of the cell cycle and the induction of DNA repair analogous to ionizing radiation [87].

Taking into account the difference in expression profiles between BLM-sensitive and BLM-insensitive individuals, these authors suggest that further studies aimed at elucidating the genes responsible for the development of sporadic cancers should be performed.

In 2008, Angelini et al. [88] investigated the possible association between genetic polymorphisms in the GSTT1, GSTM1, XPD, XRCC1 and XRCC3 genes (analyzed by PCR) and the chromosomal sensitivity to BLM in the lymphocytes of 200 healthy individuals measured by the MN test. This study showed a clear association between GSTT1-null and XPD polymorphisms and both spontaneous and BLM-induced MN frequencies, whereas the effect of the XRCC1 polymorphism was marginally significant only with regard to spontaneous MN frequency [88]. Moreover, these authors stratified the population studied according to the number of protective alleles (i.e., alleles that could have a protective effect against BLM damage): a score of 3 corresponded to those individuals homozygous for the allele with a plausible protective effect (XPD-^{751}Gln, XRCC1-Arg^{399}, XRCC3-Thr^{241}), a score of 1 corresponded to individuals homozygous for the allele with a likely non-protective effect (XPD-Lys^{751}, XRCC1-^{399}Gln, XRCC3-^{241}Met) and a score of 2 corresponded to heterozygous individuals for those alleles (intermediate protection). The GSTM1 and GSTT1 polymorphisms were dichotomized into positive genotype and null genotype, with a score of 3 and 1, respectively. Angelini et al. [88] found that an increased number of protective alleles was significantly associated with a lower frequency of BLM-induced micronucleus. Thus, the higher the number of protective alleles, the lower the sensitivity to BLM of the lymphocytes of the individuals analyzed. This finding highlights the genetic basis for BLM sensitivity, which could be a useful method for identifying genotypes that might increase susceptibility in population exposed to carcinogens. The same year, Maffei et al. [89] performed a similar study in 45 non-smoker healthy individuals, but correlating chromosomal sensitivity to BLM with a single nucleotide polymorphism (SNP A1450G) of the gene for BLM hydrolase, using the micronucleus test. They found that this polymorphism has a significant effect in predetermining individual mutagen sensitivity status [89]. Moreover, Jin et al. [90] reported an increased sensitivity to BLM (mean chromatid breaks per cell) in upper aerodigestive tract mucosa of head and neck squamous cell carcinoma patients. Also in 2008, using the BLM sensitivity test, we showed that the aircrew of international flights from Argentina (occupationally exposed to cosmic radiation) and their control group (unexposed individuals) are equally sensitive to BLM G2 clastogenic effects, since both groups exhibited a similar frequency of chromatid breaks per cell in their lymphocytes [91]. However, the aircrew sampled population was almost two times more sensitive to BLM G0 clastogenic effects than controls, which suggests that chronic exposure of aircrew to cosmic radiation increases the in vitro chromosomal sensitivity of their peripheral lymphocytes to BLM (at least in the G0 stage of the cell cycle), and that occupational exposure of flight personnel to cosmic radiation does not induce an adaptive response to this compound [91]. A year later, Scarpato et al. [92] applied the BLM sensitivity test (MN version) in autoimmune and non-autoimmune thyroid patients and found that hypothyroid patients exhibit a moderate increase in the level of spontaneous genome damage, and that autoimmune thyroid patients are less sensitive to BLM than non autoimmune patients.

In 2010, Bennett et al. [93], using chromosome painting, analyzed the chromosome damage in the lymphocytes of mothers and their newborns to determine whether smoking during pregnancy, genetic susceptibility and race are associate with CAs. They also assessed genetic susceptibility by means of the BLM sensitivity test. They found that peripheral blood lymphocytes from pregnant woman were about three times more susceptible to BLM than newborns [93]. More recently, Buchynska et al. [94] analyzed the DNA repair deficiency in peripheral blood lymphocytes of endometrial cancer patients with a family history of cancer using the BLM sensitivity test (comet assay version). They found that endometrial cancer patients are more susceptible to BLM than healthy women, and that the efficiency of DNA repair depends on the family history of cancer (i.e., patients with a family history of cancer repair less efficiently the DNA damage induced by BLM than patients with sporadic cancer). In 2015, Federici et al. [95] applied the BLM sensitivity test to individuals with pulmonary arterial hypertension and found that peripheral blood mononuclear cells from patients and relatives showed markedly sensitivity to BLM (and also to etoposide). Finally, in 2017 Kroupa et al. [96] analyzed the chromosome damage induced by BLM (chromatid breaks frequency) and its relationship with telomere length in peripheral blood lymphocytes of newly diagnosed cancer patients (with breast and colorectal cancer). These authors found that altered DNA repair (double-strand breaks) in peripheral blood lymphocytes occurs particularly in colorectal cancer, and that telomere shortening may be associated with a decreased capacity to DNA repair [96].

In summary, BLM sensitivity test, in its different versions, has been applied to several types of human cells or individuals, in most cases being useful to determine their DNA repair capacity as an indirect measure to estimate the risk of cancer.

6. BLM-induced bystander effects on mammalian cells

Physical or chemical stress applied to a cell system triggers a signal cascade that is transmitted to the neighboring cell population in a process known as 'bystander effect'. In recent years, a few studies dealt with the bystander effect of BLM in mammalian cells. Thus, in 2011, Chinnadurai et al. [97] analyzed the bystander effects induced by BLM, neocarzinostatin and ionizing radiation in normal diploid human lung fibroblasts (WI-38 cell line), bone marrow mesenchymal stem cells, lung adenocarcinoma cells (A-549 and NCI-H23 cell lines) and peripheral blood lymphocytes, using the MN test as endpoint. Bystander response was observed in all human cell types analyzed co-cultured with exposed cells, although undifferentiated bone marrow stem cells and lymphocytes showed a higher magnitude of bystander response [97]. Therefore, chemotherapeutic agents induce a bystander response similar to ionizing radiation, which is independent of the cell type. Interestingly, by using dimethyl sulphoxide (a free radical scavenger) this study showed that reactive oxygen species are very likely involved in the bystander response induced by BLM, since a significant reduction in the frequency of BLM-induced MN was observed in co-cultured bone marrow cells and lymphocytes pretreated with dimethyl sulphoxide [97]. A couple of years later, Basheerudeen et al. [98] investigated the bystander effect of BLM in human brain glioblastoma cells. To this end, these cells were exposed to BLM, and DNA damage was measured using the MN test and the γ-H2AX assay. Unexposed cells co-cultured with BLM-exposed cells did not show any significant increase in either DNA damage, cytotoxicity or a delay in cell cycle kinetics, which suggests that BLM does not induce a bystander response in brain glioblastoma cells. More recently, the bystander effects induced by BLM, were investigated by Savu et al. [99] in mouse fibroblasts. BLM induced bystander response was reflected primarily as an increased DNA damage. This was dependent on the concentration of BLM and time of media conditioning. Interestingly, they found that reactive oxygen species but not nitrogen oxide are involved in the transmission of the bystander effect [99]. In conclusion, in spite of the fact that the above studies suggest that the bystander effect of BLM and its magnitude seems to be dependent on the cell type, further studies are needed to definitely prove the bystander effect induced by BLM.

7. Effect of antioxidants and other compounds on DNA and chromosome damage induced by BLM in mammalian cells

In the last two decades, several studies showed that not only antioxidant, but also other non-antioxidant compounds prevent or even potentiate the chromosome and/or DNA damage induced by BLM in mammalian cells. Next, we will consider each one of these studies in detail.

In 1993, Chatterjee and Jacob-Raman [100] reported that pre-treatment with the aminoacid cysteine (CYST) yielded weak, non-significant protection in muntjac lymphocytes from BLM-induced chromosome damage, whereas posttreatment caused a mild potentiation of the clastogenic effect of BLM. These authors hypothesized that CYST could act as a reducing agent on BLM, thus contributing to the redox potential of this compound [100]. Alternatively, the exogenously added CYST could be incorporated into the pathway for GSH biosynthesis, triggering the elevation of intracellular GSH levels, this thiol also acting as a reducing agent for BLM [100]. A few years later, Grillo et al. [101] analyzed the effect of the phenolic antioxidant compound butylated hydroxytoluene (BHT) on the chromosome damage induced by BLM in CHO cells. Post-treatment with BHT strongly decreased the frequency of chromosome-type aberrations induced by BLM in G0/G1 and G1 and of chromatid-type aberrations induced by this compound in G2, probably by scavenging the free radicals produced by BLM, thus avoiding the formation of DNA double strand-breaks by this compound. These authors considered the effect of BHT as evidence that CAs are induced by BLM following a two-step mechanism [101]. The same year, Chattopadhyay et al. [102] studied the effect of reduced-glutathione, glutathione-ester and buthionine sulphoximine on the clastogenic activity of BLM in normal human lymphocytes. They found that depletion of endogenous glutathione (GSH) by buthionine sulphoximine reduced the clastogenic action of BLM, whereas elevation of endogenous GSH by treating the cells with GSH and GSH-ester, potentiates the cytotoxicity of BLM [102]. The observed reduction in the effect of BLM in GSH-depleted cells could be explained on the basis of the failure of reactivation of the oxidized BLM by the reducing agent GSH which is present endogenously. Similarly, free radicals generated due to reduction of oxidized BLM by the increased level of cellular GSH, after treating the cells with GSH or GSH-ester, could be responsible for the increasing frequency of CAs (deletion and chromatid breaks) [102]. In addition, as previously mentioned, Hoffmann et al. [48–51] reported that several aminothiols (including the active metabolite of amifostine, WR-1065 and CSM) potentiate the clastogenic effects of BLM (measured by the MN test) in G0 human lymphocytes. According to these authors, this effect is probably due to the activation of BLM-Fe^{+2} or the regeneration of activated BLM from inactive BLM by these thiols. These authors found that the higher the number of amino groups present in the thiol, the more effective the potentiation by these compounds is (i.e, the diamine thiol WR-1065 is more effective than the monoamine thiol CSM), since amino groups, binding to DNA, may alter its conformation, thus facilitating BLM action on DNA [51]. Similar results were obtained by Mira et al. [23] with ditiothreitol (DTT) and CSM in human lymphoblastoid cells. These authors assessed the influence of the non-protein thiols GSH, β-Mercaptoethanol (BME), DTT, CYST and CSM on the DNA damage, DNA repair, CAs and cell killing induced by BLM in human lymphoblastoid cells and found that, at the chromosomal level, GSH, BME and CYST showed a protective effect when added to cell cultures before BLM, while DTT and CSM potentiated the clastogenic effect of BLM. Moreover, at the DNA level all thiols potentiated the DNA damage induced by BLM [23]. The protective effect by BME and CYST on BLM-induced genotoxicity is not due to postreplicative DNA repair induced by these thiols, since none of them had a significant effect on the yield of chromatid-type aberrations in the G2 stage of cell cycle [23]. Moreover, the protective effect against BLM-induced clastogenic effects could be due to an increase of the intracellular level of GSH by BME and CYST [23,51], since endogenous GSH affects DNA double-strand breaks rejoining [103]. The lack of a protective effect by thiols when added after BLM treatment suggests that, once BLM reaches DNA and damages it, thiols are unable to neutralize the genotoxic effect of this compound [23]. As previously proposed by Hoffmann et al. [51], the potentiation of BLM action on DNA by thiols could be due to a reactivation of the BLM complex. Both, the amino and the thiol groups seem to be necessary to explain the potentiation effect of aminothiols on BLM genotoxicity [51]. DTT, which is a dithiol, produced the greatest potentiation of DNA damage by BLM in human lymphoblastoid cells [23], probably because of the presence of two sulphydryl groups, which reactivates BLM by reducing chelated Fe. The discrepancies observed at cytogenetic (CAs) and molecular (DNA damage) levels by treatments with GSH, BME, and CYST in BLM-exposed cells might be explained by DNA repair occurring during the last 24 h after BLM treatment in cell cultures analyzed for chromosome damage. DNA damage induced by BLM was analyzed immediately after treatments, whereas CAs were scored 24 h after treatments. Therefore, cells scored for aberrations had more time for DNA repair than cells analyzed for DNA damage.

On the other hand, Glei et al. [104] reported in 2002 that the antioxidant carotenoid beta-carotene reduces the BLM-induced DNA damage (strand breaks, as measured by the comet assay) in human lymphocytes, whereas lycopene was ineffective in doing so. However, none of these carotenoids protected these cells against endogenously arising oxidized DNA bases and has no effect on DNA repair [104]. The reason why this happens remains to be determined. Also in 2002, Buschini et al. [105] showed that amifostine (WR-2721) protects normal (human lymphocytes) but not tumor cells (K562 acute myelogenous leukemia cell line) from BLM-induced DNA damage (assessed by the comet assay). This differential effect of WR-2721 has been adscribed to the efficient dephosphorylation of aminothiol by alkaline phosphatase in lymphocytes, whereas K562 cells are unable to activate amifostine [105]. In 2004, Lee et al. [106] reported that pretreatment with genistein, one on the major Soy isoflavones with antioxidant properties, significantly decreased the frequency of MN induced by BLM in HL-60 human leukemia cells but increased the DNA damage (strand breaks measured by the comet assay) induced by the antibiotic in these cells. These authors observed a dual antagonistic effect of genistein, since it enhanced BLM-induced cytotoxicity in HL-60 cells, while it protected normal lymphocytes from the cytotoxicity of BLM. These effects could be due to a free radical scavenging action of genistein, since this compound is an effective scavenger of hydrogen peroxide. However, if genistein had direct scavenging ability, a protective effect on HL-60 cells could be expected, and this is not the case. Alternatively, genistein could protect human lymphocytes from BLM-induced damage by inducing the production of endogenous antioxidants such as GSH. If genistein had antioxidant activity by inducing endogenous antioxidants, a selective effect by different mechanisms could be expected in regards to inducing antioxidant enzymes between HL-60 and normal cells [106]. Moreover, genistein is a topoisomerase II inhibitor. As such, these authors proposed that genistein may inactivate the transcriptional factor NF-κB in HL-60 and other cancer cells, increasing the killing of cells and so the sensitivity to BLM in these cells [106]. However, BLM has been reported to activate NF-κB [107]. Clearly, further investigations are needed to clarify the differential effect of genistein on normal and cancer cells against BLM damage. The same year, Wozniak et al. [108] reported that vitamin E (alpha-tocopherol), a well-kown antioxidant, protects human colonic mucosa cells from DNA damage induced by BLM (measured by the comet assay), allowing complete reparation after 120-min post-treatment incubation of the cells. Moreover, they did not observe any protection by catalase in these cells, suggesting that hydrogen peroxide might not be involved in the production of DNA damage by BLM in these cells. This is in good agreement with previous observations by Ejchart [109] who found that endo- and exogenous catalase did not influence BLM genotoxicity (measured using the MN test and the comet assay) in different human cell lines. Moreover, in 2006, Glei et al. [110] reported that the main catechin of green tea, (−)-epigallocatechin-3-gallate, an antioxidant compound, reduces the DNA damage (strand breaks and altered pyrimidine bases, assessed by the comet assay) induced by BLM in human peripheral blood lymphocytes. This effect is probably due to a free radical scavenging activity of (−)-epigallocatechin-3-gallate. A year later, Jagetia et al. [111] reported that naringin, a grapefruit flavanone with antioxidant and metal chelating properties, protects V79 hamster cells

against BLM-induced chromosome (assessed by the MN test) and DNA (assessed by the comet assay) damage. These authors pointed out that the protective effect of naringin against BLM genotoxicity may be due to several factors, including free radical scavenging, increased antioxidant status, iron chelation, inhibition of NF-κB and apoptosis [111]. Similar results but using human lymphocytes and the MN test and CAs assay to evaluate BLM clastogenic effects, were reported by Yilmaz et al. [112] in 2016.

In 2010, Laffon et al. [113] reported that the organic selenium compound selenomethionine partially prevents the BLM-induced DNA damage (assessed by the comet assay) in human lymphocytes, an effect very likely due to its antioxidant capacity. The same year, Pinto et al. [114] reported that the bee pollen from *Cystus incanus* and *Salix alba* partially prevents the chromosome damage (assessed by the MN test) induced by BLM in human lymphocytes. This effect is probably due to the antioxidant properties of bee pollen [115]. In 2012, Sram et al. [116] in a review article about the use of vitamin C for DNA damage prevention in humans, reported that vitamin C supplementation decreases the sensitivity to BLM (chromatid breaks per cell), an effect very likely due to the antioxidant effects of this vitamin. In 2016, Cho et al. [117] reported that pretreatment with onion extract significantly decreases the BLM-induced chromosome and DNA damage (assessed by the MN test and the comet assay, respectively) in human lymphocytes. This effect is probably due to an antioxidant effect produced by the flavonoids and phenolic compounds contained in the onions [117]. The same year, Nasiri et al. [118] reported that lovastatin, a free radical scavenger, prevents BLM-induced DNA damage (assessed by the comet assay) in HepG2 cells. Very recently, Galhena et al. [119] reported that a polyherbal aqueous extract composed of *Nigella sativa* (seeds), *Hemidesmus indicus* (roots) and *Smilax glabra* (rhizome), significantly protects human lymphocytes from BLM clastogenic effects (assessed by the induction of CAs, MN and γ-H2AX foci). These effects have been ascribed to the antioxidants compounds present in the extract [119]. Since this polyherbal extract consists of a number of active ingredients, it is not possible by now to determine which of these ingredients is responsible for the antioxidant activity of the extract. Also in 2017, Mistry et al. [120] reported that aqueous and methanolic extracts from *Alstonia scholaris* bark, significantly reduce the BLM-induced chromosome damage (chromosome- and chromatid-type aberrations) in human lymphocytes. These authors pointed out that the protective effect of these extracts against BLM could be due to certain compounds present in the extract which enhance DNA repair capacity [120].

Besides the abovementioned compounds, the cytokine recombinant interferon-alpha-2a (rIFN-alpha-2a) also exhibited a protective effect against BLM-induced damage in mammalian cells [121]. In effect, a study carried out in our laboratory in 2002 showed that Recombinant IFN-alpha-2a (4500–180,000 IU/ml) added to the cell cultures 0.5 or 24 h before BLM (and left in the culture medium until the end of treatments) or immediately after BLM treatment (and left in the culture medium until harvesting) produced a significant inhibition of the yield of CAs by BLM in CHO cells [121]. We suggested that the inhibitory effect of rIFN-alpha-2a on the induction of CAs by BLM is mainly due to the stimulation of DNA synthesis and repair by the cytokine, although further studies will be needed to confirm this assumption.

Besides the above reports, indirect evidence of the involvement of free radicals in the clastogenic action of BLM was provided by an early study performed in our laboratory, in which we showed that the chromosomal sensitivity of human lymphocytes to the clastogenic effects of BLM (dicentric chromosomes) is inversely correlated with the levels of antioxidant enzymes (catalase and peroxidase in plasma, and superoxide dismutase in whole blood, erythrocytes and plasma) [122]. Thus, we suggested that by determining the levels of antioxidant enzymes in a given cell population, it would be possible to predict the chromosomal sensitivity of these cells to BLM. However, no further studies were performed to confirm this assumption.

Despite the above findings, a few reports indicated no protection at all by antioxidants and other compounds against BLM-induced chromosome and DNA damage in mammalian cells. Thus, Goodman et al. [123] showed that the dietary supplementation of healthy individuals with beta-carotene (Vitamin A) and alpha-tocopherol (Vitamin E) did not influence the sensitivity to BLM of these individuals. However, due to the limitations of the study (small sample size, i.e., 16 women and 6 men, the absence of information on the adequacy of the length of the wash-out after administration of the vitamin supplements, and the use of individuals with low mutagen sensitivity scores) [123], the conclusion of the study should be taken with caution. A year later, Cecchi et al. [124] showed that vitamin C had no significant effect on the frequency of CAs induced by BLM in human lymphocytes from smokers and non-smokers individuals. Moreover, in 2002 Oliveira et al. [125], also using the MN test, showed that the fungal metabolite wortmannin enhances the DNA damage induced by BLM in V79 hamster cells. This effect is probably due to the fact that wortmannin is a potent and irreversible inhibitor of the enzyme DNA dependent protein kinase (DNA-PK) [126], which plays a pivotal role in the repair of DNA double-strand breaks in mammalian cells. Very recently, Gowda et al. [127] reported that honokiol, a lignan isolated from the bark, seed cones, and leaves of trees belonging to the genus *Magnolia* exhibiting antioxidant properties, inhibits polymerases β and α and increases BLM sensitivity of several human cancer cell lines. These authors hypothesized that, by inhibiting DNA polymerases, honokiol also inhibits DNA repair, thus potentiating the DNA damage effect of BLM [127]. Thus, most of the studies reviewed here concerning the effects of antioxidant compounds on the genotoxicity of BLM show that these compounds inhibit the chromosome and DNA damage induced by BLM in mammalian cells, thus supporting the involvement of free radicals or active oxygen species in the genotoxicity of BLM [23,101,102,104,106,110–114,116–122]. Free radicals also seem to play some role in the still not fully proven bystander response [97,99] induced by this compound in mammalian cells. However, a few reports [108,109] suggest that H_2O_2 could not be involved in the genotoxicity of BLM, and others [123–125,127] show that some antioxidants potentiate the clastogenic and DNA damaging action of this compound. This latter effect depends on the agent that potentiates BLM-induced damage, and could be due to different mechanisms, including activation of BLM-Fe^{2+}, regeneration of activated BLM from inactive BLM-Fe^{3+}, number of amino and/or thiol groups in the compound that potentiates the damage, inhibition of DNA-PK, inhibition of DNA polymerases, etc.

8. Conclusions

Several conclusions can be drawn from the studies on BLM genotoxicity in mammalian cells developed since the publication of the review article by Povirk and Finley Austin [2]. Concerning DNA damage, this antibiotic induces nuclear DNA fragmentation, and damages telomeric and mtDNA. Also, human telomeric DNA is a major target for BLM, and actively transcribed genes are preferentially cleaved by this compound compared with non-transcribed genes, being 5′-GT* dinucleotide sequences (where the asterisk indicates the BLM cleavage site) the preferred site of BLM breakage in human cells. In the case of mtDNA, this compound cleaves preferentially at 5′-TGT*A-3 DNA sequences (where * is the cleavage site). Despite the above findings, further studies are needed to fully understand the effects of BLM on mtDNA in mammalian cells.

Regarding the clastogenic effects of BLM on mammalian cells, the studies performed in the last decades show that this compound induces not only classic aberrations (like chromatid- or chromosome-type breaks or dicentrics) but also complex CAs (involving three or more breaks in two or more chromosomes), ICE (which implies breakage events at the chromosome ends) and other telomeric-related aberrations (such as telomere duplication or fusion). Telomere FISH also showed that in BLM-exposed cells, interstitial fragments and ICE are common aberrations (interstitial fragments seems to be a characteristic

signature of the clastogenic effect of BLM and also low-LET radiation) [61], and that telomere-related aberrations can persist several generations after treatment. Overall, the cytogenetic and molecular studies on the effects of BLM on mammalian telomeres reviewed here suggest that telomeres are more affected to BLM-induced damage not only because they are at the end of the chromosomes, but also because telomeric DNA is a major target for this compound, at least in human cells. Further molecular studies in other mammalian cells will help to reach a more definitive conclusion in this regard.

Moreover, BLM can alter ITSs, producing breakage, amplification and translocation of these telomeric-like sequences. Despite all the studies performed so far, the long-term effects of BLM on telomeres and ITSs should be further investigated in more detail and in other cell types to establish more general conclusions about the long-term clastogenic and DNA damaging effects of this compound in mammalian cells. This is important to understand the genomic instability associated with chemotherapy regimens using BLM as anticancer drug.

On the other hand, while BLM has been proven to be useful to detect an adaptive response to ionizing radiation or radiofrequency fields, further studies will be needed to fully elucidate if an adaptive response to BLM exists in mammalian cells and the possible factors that influences it. Moreover, the scarcity of data available about the bystander effect by BLM does not allow to establish definitive conclusions about it.

Although the classic BLM sensitivity test (i.e., G2-induced chromatid breaks per cell) has been applied to several systems, and in most cases has been useful to determine the DNA repair capacity of cells or individuals, more studies are needed to validate the alternative versions of this test developed in recent years.

Finally, most of the studies concerning the effects of antioxidant compounds on the genotoxicity of BLM developed in the last decades support the involvement of free radicals or active oxygen species in the chromosome and DNA damage and probably in the bystander response induced by this compound in mammalian cells. However, further studies are needed to confirm the role of free radicals in the bystander effect of BLM and the involvement of H_2O_2 in the genotoxicity of BLM.

Conflict of interests

The authors declare that there are no conflicts of interest.

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