Environmental and Molecular Mutagenesis 54:147–152 (2013)

Brief Communication

Interstitial Telomeric Sequences Are Not Preferentially Involved in the Chromosome Damage Induced by the Methylating Compound Streptozotocin in Chinese Hamster Cells

Ivana Y. Quiroga, Natalia S. Paviolo, and Alejandro D. Bolzán*

Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CCT-CONICET La Plata-CICPBA), La Plata, Argentina

The effect of the methylating compound streptozotocin (STZ) on interstitial telomeric sequences (ITSs) was investigated in Chinese hamster ovary (CHO) cells by using peptide nucleic acid-fluorescence in situ hybridization with a pantelomeric probe. Cells were exposed to increasing concentrations of STZ, and chromosomal aberrations were analyzed at the first mitosis after treatment. The frequency of chromosomal aberrations directly involving ITSs increased in STZ-treated cells by a factor of 2.6 (2 mM) and 3.6 (4 mM) when compared with the frequency of these aberrations in control cells ($P < 0.05$). However, no significant differences were found between control and exposed cells in the percentage of aberrations directly involving ITSs, demonstrating that these repeat regions were not preferentially involved in the chromosome damage induced by STZ. In addition, STZ did not alter telomerase activity, suggesting that this enzyme may not be involved in the induction of chromosomal aberrations by this compound. Environ. Mol. Mutagen. 54:147–152, 2013.

Key words: telomere; chromosomal aberrations; telomere PNA-FISH; CHO cells; alkylating compounds

INTRODUCTION

By definition, telomeric repeats are located at the ends of eukaryotic chromosomes. However, some vertebrate species show large blocks of (TTAGGG)*n* repeats outside of the chromosome ends. These repeats are called interstitial telomeric sequences (ITSs) and comprise repeats located close to the centromeres and at interstitial sites, that is, between the centromeres and the telomeres (for review, see Lin and Yan, 2008; Ruíz-Herrera et al., 2008). Experimental evidence shows that chromosomal regions containing ITSs are prone to chromosome breakage, fragility, and recombination [Bolzán and Bianchi, 2006; Lin and Yan, 2008; Ruíz-Herrera et al., 2008; Bolzán, 2009, 2012]. Heterochromatic ITSs are especially sensitive to breakage, and in Chinese hamster cells, these sequences extend for hundreds of kilobases on each chromosome and constitute a major part of satellite sequences [Slijepcevic and Bryant, 1995].

It has been pointed out that the involvement of ITSs in the induction of chromosomal aberrations depends on multiple factors, including the type of aberration, the nature of ITSs, and the type of clastogen [Lin and Yan, 2008]. Therefore, to clearly elucidate the relationship between heterochromatic ITSs and chromosome damage, different kinds of clastogens (i.e., physical and chemical, S-independent and S-dependent) must be tested on cells containing these sequences. Previously, we investigated the effects of two radiomimetic compounds, bleomycin and streptonigrin, on ITSs from Chinese hamster ovary (CHO) cell lines [Bolzán et al., 2001; Sánchez et al., 2009, 2010]. We found that radiomimetic compounds are capable of inducing translocation and amplification of ITSs [Bolzán et al., 2001; Sánchez et al., 2009, 2010] and also breakage at centromeric regions rich in ITSs; however, these chromosome regions are not the preferential target for the clastogenic action of these compounds [Sánchez et al., 2009, 2010]. More recently, we showed

Grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); Grant Number PIP No. 0316; Grant sponsor: Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA).

*Correspondence to: Alejandro D. Bolzán, Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CCT-CONICET La Plata-CICPBA), C.C. 403, 1900 La Plata, Argentina. E-mail: abolzan@imbice.org.ar or adbolzan64@gmail.com

Received 6 July 2012; provisionally accepted 5 October 2012; and in final form 10 October 2012

DOI 10.1002/em.21750

Published online 28 November 2012 in Wiley Online Library (wileyonlinelibrary.com).

that bleomycin and streptonigrin induce delayed instability of ITSs in CHO cells in the form of additional telomere FISH signals [Mencucci et al., 2012; Vidal Bravo et al., 2012]. In this study, we analyzed the relationship between ITSs and chromosomal aberrations induced by streptozotocin (STZ) in CHO cells 18 h after treatment to obtain further insight into the short-term effects of chemical mutagens on ITSs. Because of the presence of large blocks of heterochromatic ITS at centromeric regions on most of the chromosomes, and the fact that about 95% of the telomeric FISH signals in a CHO metaphase cell correspond to ITS, these cells represent an ideal choice for analyzing the behavior of ITS in clastogen-exposed cells [Balajee et al., 1994; Bolzán et al., 2001; Sánchez et al., 2009, 2010].

STZ is an antibiotic isolated from *Streptomyces achromogenes*, which is usually used to experimentally induce diabetes mellitus in laboratory animals, and it has been considered a potential compound for the clinical treatment of some malignant diseases (for review, see Bolzán and Bianchi, 2002). STZ is a potent alkylating agent that directly methylates DNA, giving rise to chromosome and DNA damage [Bolzán and Bianchi, 2002]. STZ exerts its clastogenic effect mainly in an S-dependent manner, inducing both chromatid- and chromosome-type aberrations [Bolzán and Bianchi, 2002]. Although the effects of STZ on telomeres from mammalian cells are partially known [Bolzán and Bianchi, 2005], there is no data available concerning those on ITSs. In this work, we found that STZ induces chromosome damage in ITSs as well as in any other part of the genome of CHO cells, which shows that these sequences are not preferentially involved in induced chromosomal aberrations.

MATERIALS AND METHODS

Cell Culture, Drug Treatments, and Cell Harvesting

The CHO cell line was obtained from the Instituto Multidisciplinario de Biología Celular cell repository (La Plata, Argentina) and is an established immortalized cell line derived from CHO cells possessing on average $2n = 21$ [Bolzán et al., 2001; Sánchez et al., 2009, 2010]. CHO cells were grown in Ham's F10 medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO_2 atmosphere. Cells were cultured as monolayer in TC25 Corning flasks containing 1.5×10^5 cells per milliliter. During the log phase of growth, the cells were treated for 30 min with STZ (CAS No. 18883-66-4, Sigma) at the concentrations indicated below. The concentrations used were based on the previous studies carried out in our laboratory [Bolzán et al., 1998, 2000]. As STZ is unstable at pH 7 [Herr et al., 1967], it was prepared immediately before use by dissolving the drug in 0.02 M sodium citrate (pH 4.4). At the end of the pulse treatment with STZ or sodium citrate, the cells were washed twice with Hank's balanced salt solution and kept in culture with fresh culture medium containing 10 μg/ml BrdUrd (CAS No. 59-14-3, Sigma) until harvesting. The cultures were harvested 18 h after the end of treatment to determine the chromosome damage induced by STZ in first metaphase cells after treatment. During the last 2.5 h of culture, cells were exposed to colchicine (0.1 μg/ml; CAS No. 64-86-8, Sigma). Chromosome preparations were made following standard procedures. After harvesting, cells were hypotonically shocked, fixed in methanol:acetic acid (3:1), spread onto glass slides, and processed for peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH). 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 [Perry and Wolff, 1974]. Briefly, the slides were stained with Hoechst 33258 (1 μg/ml; CAS No. 23491-45-4, Sigma) in 0.1 M phosphate buffer (pH 6.8) for 30 min, exposed to UV (365 nm) for 1 h, and stained with Giemsa.

FISH with the PNA Pantelomeric Probe (PNA-FISH) and Chromosome Analysis

A Cy3-conjugated PNA pantelomeric probe [Cy3-$(CCCTAA)_3$] obtained from Panagene (Korea) was used. FISH was performed according to the protocol provided by the supplier and as described elsewhere [Paviolo et al., 2012]. The slides were mounted on an antifade reagent containing DAPI (4,6-diamidino-2-phenylindole) as counterstain. Fluorescence microscopy was performed on a Nikon Eclipse 50i epifluorescence microscope equipped with an HBO 100 mercury lamp, a Nikon high-resolution digital color camera (DS-Ri-U3), and filters for DAPI and Cy3 (Chroma Technology, Rockingham, VT).

Scoring of Aberrations

Chromosome analysis was performed on coded slides. Cell cycle analysis made on 100 cells from each sample using the FPG technique [Perry and Wolff, 1974] showed that, in all cases, 95–100% of cells were in the first division after treatment. Therefore, no combination of FISH plus FPG was necessary for aberration analysis. Centromeres were identified using the DAPI filter, whereas telomeric signals were observed using the Cy3 filter. To obtain the final, two-color FISH images, DAPI and Cy3 images were merged using the NIS-Element Imaging Software 3.22 (Nikon). In the CHO cell line available in our laboratory, the first two pairs of chromosomes of the karyotype exhibit absolutely no telomeric signals, whereas the rest of the chromosomes show telomeric signals in the centromeric region. The only exceptions being one chromosome that also exhibits an interstitial telomeric signal at its long arm, and one chromosome that exhibits telomeric signals at one of their ends. Moreover, some cells contain an additional chromosome with terminal signals [Bolzán et al., 2001; Sánchez et al., 2009, 2010] (Fig. 1A). As the position and number of centromeres could be easily determined with DAPI staining, all types of unstable aberrations were scored, namely, (1) dicentric chromosomes; (2) centric rings; (3) acentric fragments (without discrimination of their type); (4) chromatid-type aberrations: breaks and exchanges (triradials and quadriradials); and (5) centromeric breaks. Gaps were excluded from the analysis. The frequencies of aberrations with and without telomeric signals, and the number and distribution of telomeric repeats in each of the aberrations analyzed were determined in untreated (control) and STZ-treated cells. In addition, we scored those aberrations directly involving ITSs, that is, centromeric breaks exhibiting dissociation of the telomeric signal, translocation of ITSs, additional telomeric FISH signals, labeled acentric fragments, and monochromatid and isochromatid breaks and chromatid exchanges exhibiting telomeric signal at the site of breakage [Bolzán, 2009, 2012]. Additional telomeric FISH signals indicate telomeric signals not present in the standard karyotype of CHO cells after PNA telomeric FISH [Bolzán et al., 2001]. One hundred metaphases per sample per experiment were analyzed for the presence of aberrations. Two independent experiments were carried out.

Mitotic Index

The mitotic index (MI) was estimated for each sample and treatment by counting 1,000 cells and by recording the number of cells at metaphase, and then MI was calculated by applying the following formula:

$$\text{MI (\%)} = (\text{Number of cells in division/Total number of cells}) \times 100.$$

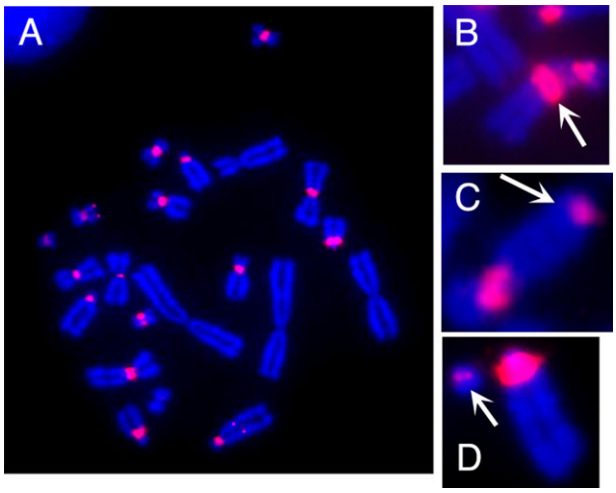

Fig. 1. Patterns of hybridization of (TTAGGG)*n* repeats in the metaphase spreads from untreated (**A**) and STZ-treated (**B–D**) CHO cells after PNA-FISH with a telomeric probe. A: Control metaphase ($2n = 21$). Note the presence of telomeric signals at the centromeric regions of all chromosomes, except the largest pairs (i.e., Pairs 1 and 2), one interstitial signal in the long arm of chromosome 4, and terminal signals in one small metacentric chromosome. B–D: Different types of chromosomal aberrations directly involving ITSs observed in STZ-exposed cells: (B) new (double) interstitial telomeric signal in the long arm of an acrocentric chromosome (arrow indicates the site of the additional signal); (C) new terminal telomeric signal in one arm of an acrocentric chromosome (arrow indicates the site of the additional signal); and (D) small acentric fragment labeled with the telomeric probe (arrow). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Telomerase Activity Assay

Telomerase activity was measured in all samples by using the TRAPeze-RT telomerase detection Kit (Millipore, Bedford, MA) and Taq Platinum (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The reactions were performed in two independent experiments, each one in duplicate. This assay quantifies telomerase activity by measuring real-time fluorescence emission using quantitative PCR and was performed as described elsewhere [Paviolo et al., 2012]. Telomerase activity (total product generated) was calculated by comparing the average *Ct* values from the sample wells against the standard curve generated by the TSR8 control template. The assays were performed on a real-time fluorescence capable thermocycler with FAM detection ABI Prism 7500 (Bio-Rad Laboratories, Irvine, CA).

Statistical Analysis of Data

Statistical analysis of data was performed using GraphPad Prism version 4.00 software for Windows (GraphPad Software, San Diego, CA). The comparisons between control versus exposed cultures in the percentage of aberrant metaphases and in the percentage of cells exhibiting telomeric signals were carried out using the χ^2 test. The significance of differences in aberration frequencies among different treatments was obtained by comparing the *Z*-score of Poisson distributions of observed and expected values with 95% confidence intervals [Fisher and Yates, 1957], as data were distributed following a Poisson distribution. The Kruskal-Wallis test was used to determine the statistical significance of the differences in MI and in telomerase activity between STZ-treated and control cells. Differences were considered to be statistically significant at two-sided *P* values < 0.05.

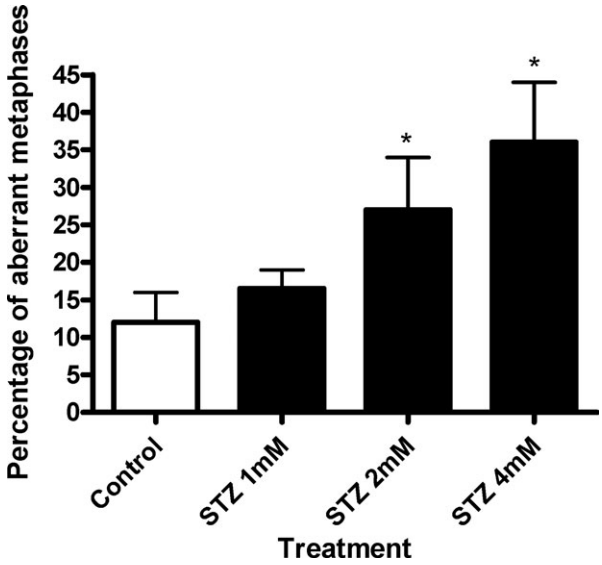

Fig. 2. Percentage of aberrant metaphases in untreated and STZ-exposed CHO cells analyzed 18 h after treatment. Data represent mean values from two independent experiments (100 metaphase cells per sample per experiment analyzed). For each treatment, mean ± SE is indicated. *χ^2 test indicated significant increase in the percentage of damaged cells induced by STZ ($P < 0.05$). Data from control cultures exposed to the solvent (0.02 M sodium citrate) were excluded from the figure as no significant differences in the percentage of aberrant metaphases were found between cultures with and without the solvent ($P > 0.05$).

RESULTS

Distribution of Telomeric Sequences in Untreated CHO Cells

In good agreement with previous reports [Bolzán et al., 2001; Sánchez et al., 2009, 2010; Mencucci et al., 2012; Vidal Bravo et al., 2012], cytogenetic analysis of untreated cultures of CHO cells after telomere PNA-FISH showed telomeric repeats, predominantly localized in the centromeric regions of the chromosomes (Fig. 1A). Very few metaphases also exhibited a second chromosome with telomeric signals at one end, as previously reported [Bolzán et al., 2001]. Moreover, single telomeric signals randomly distributed (located in different chromosomes and different chromosome arms) were also observed in control cells at a very low frequency (0.03 per cell).

Distribution of Telomeric Sequences in the Chromosomal Aberrations Induced by STZ in CHO Cells

The analysis of the percentage of aberrant cells (i.e., metaphases showing at least one aberration) and the frequency of total chromosomal aberrations showed that STZ induced a significant increase in both parameters at the concentrations of 2 and 4 mM ($P < 0.05$; Figs. 2 and 3A and Table I). No significant differences in the percentage

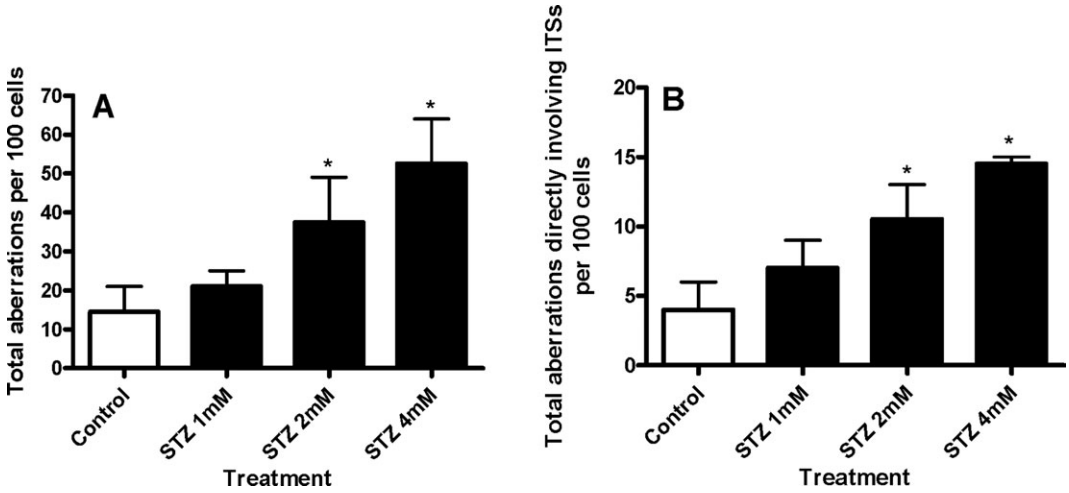

Fig. 3. **A**: Induction of chromosome damage by STZ (all aberrations included; see data in Table I) in CHO cells analyzed 18 h after treatment. Data represent mean ± SE values from two independent experiments. The *Z*-score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by STZ when compared with untreated (control) cultures ($P < 0.05$). **B**: Changes in the frequency of chromosomal aberrations directly involving ITSs (i.e., instability of ITSs; see Table I) in CHO cells as a function of STZ concentration 18 h after treatment. Data represent mean ± SE values from two independent experiments. The *Z*-score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by STZ when compared with untreated (control) cultures ($P < 0.05$). Data from control cultures exposed to the solvent (0.02 M sodium citrate) were excluded from the figure as no significant differences in the frequencies of aberrations were found between cultures with and without the solvent ($P > 0.05$).

of aberrant cells or in the frequencies of chromosomal aberrations between control cultures and cells exposed to the solvent of STZ alone (i.e., sodium citrate) were found ($P > 0.05$; data not shown). A detailed analysis of the type of aberrations induced by STZ showed that this compound induced mainly chromatid and chromosome breaks (including those occurring at centromeric regions) and acentric fragments in CHO cells analyzed 18 h after treatment (Table I). In addition, we determined the frequency of chromosomal aberrations directly involving ITSs, which includes all the aberrations where ITSs are involved in the breakage event (see Materials and Methods section). Furthermore, almost 100% of the telomeric FISH signals in a CHO metaphase cell corresponds to ITSs [Balajee et al., 1994; Bolzán et al., 2001; Sánchez et al., 2009, 2010], and those very few telomeric signals located at one end of one or two chromosomes of the CHO karyotype represent former ITSs that had been converted into new telomeres, either by chromosome breakage or by amplification mechanisms [Slijepcevic et al., 1996]. According to these data, it can be assumed that the telomeric sequences involved in the chromosomal aberrations induced by STZ, which directly affect ITSs, come from broken ITSs and not from terminal telomeric sequences. Moreover, the CHO chromosomes (one or, less frequently, two) with terminal signals at one end were always present in cells possessing additional signals, and therefore, these new telomeric signals were considered as ITSs.

We found that at high concentrations (2 and 4 mM), STZ induces a significant increase in the frequency of total aberrations directly involving ITSs in CHO cells analyzed 18 h after treatment ($P < 0.05$; Table I and Fig. 3B; see Figs. 1B and 1C for illustrations of additional FISH signals, and Fig. 1D for illustration of a labeled acentric fragment). The frequency of chromosomal aberrations directly involving ITSs increased in STZ-treated cells by a factor of 2.6 (2 mM) and 3.6 (4 mM) when compared with the frequency of these aberrations in control cells ($P < 0.05$; Fig. 3B). Table I shows that in both control cultures and STZ-treated cells, chromosomal aberrations directly involving ITSs represent 28-33% of the total aberrations observed, suggesting that these repeat regions were not preferentially involved in aberration formation ($P > 0.05$, χ^2 test).

Finally, no significant differences were found between treatments with regard to MI ($P > 0.05$; data not shown).

Effect of STZ on Telomerase Activity in CHO Cells

Statistical analysis showed that the level of telomerase activity in STZ-treated cells was very similar to that of the control (unexposed) cells with or without sodium citrate ($P > 0.05$; data not shown).

DISCUSSION

In this work, we report that exposure of CHO cells to STZ does not preferentially affect ITSs and that telomerase may not be involved in the short-term clastogenic action of this compound.

Our present findings showing a significant induction of chromosome damage by STZ in CHO cells analyzed 18 h

TABLE I. Chromosomal Aberrations Observed in Untreated and STZ-Exposed CHO Cells After PNA Telomeric FISH Scoring

Treatment	Dic	R	AF	Breaks	Chromatid exchanges	Centromeric breaks	T	ATS (●/●●)	Total aberrations (frequency per cell)	Aberrations directly involving ITSs
Control	1 (0)	0	2 (1)	14 (0)	0	6 (1/3)	0	6 (6/0)	29 (0.14)	8 (27.6%)
STZ 1 mM	2 (2)	0	10 (3)	8 (0)	2 (0)	10 (1/3)	1	9 (5/4)	42 (0.21)	14 (33.3%)
STZ 2 mM	1 (0)	0	21 (3)	30 (3)	3 (0)	7 (2/0)	0	13 (10/3)	75[a] (0.37)	21[a] (28.0%)
STZ 4 mM	8 (8)	1 (1)	24 (7)	25 (1)	6 (1)	25 (4/10)	0	16 (12/4)	105[a] (0.52)	29[a] (27.6%)

Harvesting time: 18 h after treatment. The total number of aberrations and the number (in parenthesis) of aberrations exhibiting one or more FISH signals per treatment are indicated. In the case of dicentrics (Dic) and centric rings (R), this refers to centromeric signals, whereas in the case of breaks and chromatid exchanges, this refers to the presence of signal at the site of breakage; AF, acentric fragments. Labeled centromeric breaks include breaks with (in the first place) and without (in the second place) dissociation of the telomeric signal. T, translocations of ITSs. ATS, additional telomeric FISH signals (● = single signals; ●● = double signals). In the case of aberrations directly involving ITSs (excludes dicentrics, rings, and centromeric breaks without dissociation of the telomeric signal), the percentage of aberrations with regard to total aberrations observed is indicated between brackets. A total of 200 metaphase cells per treatment were analyzed. The results of two independent experiments were pooled.

[a]Statistically significant differences between control and STZ-treated cultures ($P < 0.05$, Poisson distribution). Data from control cultures exposed to the solvent (0.02 M sodium citrate) were excluded from the table as no significant differences in the frequencies of aberrations were found between cultures with and without the solvent ($P > 0.05$).

after treatment are in accordance with previous reports, which demonstrate that this methylating compound not only induces mainly chromatid-type aberrations (like most chemical mutagens) but is also capable of inducing chromosome-type aberrations in first-mitosis cells after treatment [Capucci et al., 1995a,b,c; Bolzán et al., 1998, 2000; Bolzán and Bianchi, 2005]. In addition, by using PNA-FISH with a pantelomeric probe, we demonstrate for the first time that STZ (4 mM) induces centromeric breaks, half of them (14/25 = 56%) involving chromosomes with centromeric regions rich in ITSs. However, most of them (10/14 = 71%) do not involve dissociation of the FISH signal, which indicates that ITSs are not preferentially involved in the formation of these aberrations.

We previously reported that STZ induces telomere instability in the form of chromosomal incompleteness (i.e., chromosome end loss) in other Chinese hamster cell lines [Bolzán and Bianchi, 2005]. In this work, we found that STZ also induces instability of ITSs; however, the percentage of aberrations involving these telomeric sequences did not differ between treatments, which show that ITSs are not preferentially involved in the chromosome damage induced by this compound in CHO cells. In other words, chromosome regions containing ITSs are as sensitive to STZ as chromosome regions devoid of these telomeric sequences. In contrast, ITSs have been found to be preferentially involved in the chromosomal aberrations induced by some restriction enzymes [Balajee et al., 1994] as well as many other chemical mutagens including mitomycin C [Fernández et al., 1995], teniposide (VM-26) [Fernández et al., 1995], bleomycin [Sánchez et al., 2009], and streptonigrin [Sánchez et al., 2010]. Thus, these results confirm that the involvement of ITSs in the chromosomal aberrations induced by chemical mutagens depends on the type of clastogen (i.e., S-dependent or S-independent). Moreover, the current finding that STZ did not modify telomerase activity 18 h after treatment is in good agreement with our previous observations in bleomycin- and streptonigrin-exposed CHO cells [Bolzán et al., 2001], and shows, for the first time, that this compound has no effect on telomerase activity, at least in the short-term exposure.

In summary, our data indicate that although STZ is able to induce chromosomal aberrations involving ITSs, these telomeric sequences do not play a significant role in the clastogenic action of this compound, at least in terms of short-term exposure, as STZ induces breaks in ITSs as well as in any other part of the genome. The reason why this happens remains to be established and denotes a different mode of action on ITSs by an alkylating compound (STZ; current work) when compared with radiomimetic compounds (bleomycin or streptonigrin) [Sánchez et al., 2009, 2010]. In addition, our data suggest that telomerase is not involved in the clastogenic action of STZ in mammalian cells.

Given the critical role of telomeric sequences in genome stability and that STZ is widely used in the laboratory (as a diabetogenic agent) as well as for the clinical treatment of pancreatic endocrine tumors, we consider that the effects of this antibiotic on ITSs should be further investigated. Future studies on this area of research should aim to investigate the long-term effects of STZ on ITSs in order to determine whether this compound is able to induce delayed chromosomal instability involving ITSs, as previously observed for other antitumor antibiotics in CHO cells [Mencucci et al., 2012; Vidal Bravo et al., 2012], and telomere dysfunction (i.e., telomere FISH signal loss or duplication), as we recently observed in rat cells exposed to bleomycin [Paviolo et al., 2012].

AUTHOR CONTRIBUTIONS

A.D. Bolzán designed the study. I.Y. Quiroga, N.S. Paviolo, and A.D. Bolzán performed the experiments and collected the data. I.Y. Quiroga and A.D. Bolzán analyzed the data and prepared draft figures and tables. A.D. Bolzán, I.Y. Quiroga, and N.S. Paviolo prepared the manuscript draft. All authors approved the final manuscript. A.D. Bolzán, I.Y. Quiroga, and N.S. Paviolo had complete access to the study data.

ACKNOWLEDGMENTS

The authors thank Julieta Parisi and Daniel Castrogiovanni for technical assistance and Dr. Jorge López Camelo for assistance with the statistical analysis of data.

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Accepted by—
M. Honma