Mutation Research 760 (2014) 16–23

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

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Community address: www.elsevier.com/locate/mutres

The radiomimetic compound streptonigrin induces persistent telomere dysfunction in mammalian cells

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ARTICLE INFO

Article history:
Received 6 September 2013
Received in revised form 7 November 2013
Accepted 24 November 2013
Available online 6 January 2014

Keywords:
Streptonigrin
Incomplete chromosomes
Telomere instability
Telomere dysfunction
Long-term clastogenic effect
Mammalian cells

ABSTRACT

We analyzed the chromosomal aberrations involving telomeres in the progeny of mammalian cells exposed to the radiomimetic compound streptonigrin (SN) in order to determine if this antineoplastic drug induces long-term telomere instability. To this end, rat cells (ADIPO-P2 cell line, derived from adipose cells from Sprague–Dawley rat) were treated with a single concentration of SN (100 ng/ml), and chromosomal aberrations were analyzed 18 h and 10 and 15 days after treatment by using PNA-FISH with a pan-telomeric probe [Cy3-$(CCCTAA)_3$] to detect (TTAGGG)n repeats. Cytogenetic analysis revealed a higher frequency of telomere dysfunction-related aberrations (additional telomeric FISH signals, extra-chromosomal telomeric FISH signals, and telomere FISH signal loss and duplications) in SN-exposed cultures vs. untreated cultures at every time points analyzed. The yield of SN-induced aberrations remained very similar at 18 h, 10 days as well as 15 days after treatment. Thus, our data demonstrate that SN induces persistent telomere dysfunction in mammalian cells. Moreover, we found that the level of telomerase activity in SN-treated cells was significantly lower (up to 77%) than that of untreated control cells at each time points analyzed. This fact suggests that telomerase could be involved in SN-induced telomere dysfunction.

1. Introduction

Since telomeres play a fundamental role in maintaining genomic stability [1–4], the study of the involvement of telomeric sequences is of great importance to understand the chromosomal instability in the long term which is induced by antineoplastic drugs. This plays an important role in understanding the genomic instability associated with chemotherapy regimens.

Although it has been reported that exposure to both, sparsely [5–8] and densely [9] ionizing radiation induces persistent or delayed telomere instability in human cells, data on the long-term effects of chemical mutagens on telomeres and interstitial telomeric sequences are scarce [10–12]. These data indicate that the radiomimetic compounds bleomycin [11] and streptonigrin (SN) [10] induce delayed instability of interstitial telomeric sequences in Chinese hamster ovary (CHO) cells. Both chemical mutagens promote the appearance of additional telomeric FISH signals and/or chromosome breaks at centromeric regions rich in heterochromatic interstitial telomeric sequences 6 days after exposure. In addition, we recently showed that bleomycin induces persistent and delayed telomere instability in rat cells [12], which exhibit chromosomal abnormalities related to telomere dysfunction, such as telomere loss and duplications 10 days after treatment. These cells also show incomplete chromosomes, a type of aberration which does not involve telomere dysfunction, 10 days after exposure to bleomycin.

In order to gain further insight into the long-term effects of radiomimetic compounds with antineoplastic properties on mammalian telomeres, in our current study we investigated the induction of chromosomal aberrations involving telomeres in rat cells exposed to SN. This compound is an aminoquinone antitumor antibiotic isolated from cultures of *Streptomyces flocculus*, which shows antitumor activity against a broad range of tumors, including breast, lung, head and neck cancer, lymphoma and melanoma [13]. Although its use in cancer therapy is very limited because it induces severe and prolonged bone marrow depression [13], it has been shown that SN plays an important role in inhibiting β-catenin/T-cell factor (Tcf) signaling in human carcinogenesis. Therefore, SN is of great interest as a leading compound for chemotherapeutic treatment against β-catenin-activated tumorigenesis [14].

Despite being considered a radiomimetic compound, SN is capable of producing chromosome damage both by S-independent and S-dependent mechanisms [13]. In effect, like S-independent clastogens, SN produces chromatid-type aberrations in the G2-phase and in replicated S-phase chromatin, and chromosome-type aberrations in the G1-phase and in unreplicated S-phase chromatin. However, like S-dependent agents, SN also produces

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http://dx.doi.org/10.1016/j.mrfmmm.2013.11.009

chromatid-type aberrations in the G1-phase. It is an efficient inducer of SCEs, and has a delayed clastogenic effect as well [13,15–17]. Moreover, SN causes inhibition of topoisomerase II by stabilizing the trans-esterification intermediate of the enzyme (called cleavable complex), and thus produces DNA damage [13]. The induction of short-term telomere instability by this compound (in the form of incomplete chromosome elements) was previously demonstrated by our group [18]. No data are yet available on the long-term effects of SN on telomeres.

To accomplish our goal, we exposed rat cells to a single pulse of SN and determined the type and frequency of chromosomal aberrations at 18 h (first mitosis after exposure) and 10 and 15 days after treatment by using PNA-FISH with a pan-telomeric probe. Our data indicate that SN induces persistent telomere dysfunction in mammalian cells, in the form of additional telomeric FISH signals, extra-chromosomal telomeric FISH signals, and telomere FISH signal loss and duplication, which persists for several generations after exposure in the SN-treated cells**.** Moreover, our data suggest that telomerase could be involved in SN-induced telomere dysfunction.

2. Materials and methods

2.1. Cell culture, drug treatments and cell harvesting

The present work was carried out using a fibroblast-type cell line named ADIPO-P2 obtained from the Instituto Multidisciplinario de Biología Celular (IMBICE, La Plata, Argentina) cell repository, and established by Daniel Castrogiovanni (Cell Culture Section, IMBICE). ADIPO-P2 cells ($2n = 42–43$ chromosomes) are derived from dedifferentiated adipose cells from Sprague–Dawley rats. ADIPO-P2 chromosomes exhibit strong telomeric signals at their ends and do not show interstitial telomeric signals after telomere PNA-FISH [12]. In this way, telomere aberrations can be easily identified, with no interference of interstitial telomeric signals in the results obtained, i.e., all of the data concerning telomeric sequences instability can be ascribed to telomere instability.

ADIPO-P2 cells were grown in D-MEM high glucose medium (Gibco®, Grand Island, NY, USA) supplemented with 20% 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/ml. For each experiment, two flasks were set up, one for the control and one for the treated culture. During the log phase of growth ADIPO-P2 cells were treated with a 20 min pulse of 100 ng/ml of SN (Sigma, CAS no. 3930-19-6), dissolved in sterile Hank's balanced salt solution). Control cultures were set up in parallel but not exposed to SN. Time of exposure and concentration of SN were chosen according to previous studies carried out in our laboratory with mammalian cells exposed to this compound [11,16–19]. At the end of the pulse treatment with SN, the cells were washed twice with Hank's balanced salt solution and kept in culture with fresh culture medium until harvesting. Both control and SN-treated cells were continuously maintained in culture during seven passages or subcultures after treatment. Subcultivation was carried out whenever the cultures became confluent (approximately 4×10^5 cells/ml of culture medium). To estimate cell growth, at the time of subcultivation cells were collected by trypsinization, an aliquot of about 200 μl stained with 0.4% trypan blue, and the number of viable cells (cells not stained) was manually determined using a Neubauer chamber. Cells were then suspended in fresh culture medium and dispensed into new culture flasks containing 1×10^5 cells/ml to continue growing. The rest of the cells was discarded or dispensed in another flask for cytogenetic analysis, which was performed at 18 h and 10 and 15 days after the end of treatments. To analyze chromosomal aberrations, colchicine (0.1 μg/ml) (Sigma, CAS No. 64-86-8) was added to cell cultures during the last 3 h of culture. 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 PNA-FISH. Two independent experiments were carried out.

2.2. Fluorescence in situ hybridization with the PNA pantelomeric probe (PNA-FISH) and chromosome analysis

A Cy3-conjugated PNA pan-telomeric probe [Cy3-$(CCCTAA)_3$] obtained from Panagene (Korea) was used. FISH was performed according to the protocol provided by the supplier. Briefly, after pre-treatment with 3.7% formaldehyde and a solution containing 2 mg/ml proteinase K for 10 min, sample DNA was denatured at 85 °C for 10 min under a coverslip in the presence of the Cy3-conjugated probe. Hybridization in a moist chamber (1 h at room temperature) was followed by two washes in 2X SSC 0.1% tween 20 for 10 min at 55–60 °C. Afterwards, 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 Corp., Rockingham, VT, USA).

2.3. Scoring of aberrations

Chromosome analysis was performed on coded slides. For accurate observation and analysis, all images were digitalized and, when necessary, DAPI signals were enhanced for optimal contrast. This allowed detection of even the smallest acentric fragments present in the aberrant metaphases. 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 Corporation). The total number of centromeres and telomeric signals was counted and all unstable chromatid- and chromosome-type aberrations were scored. Since 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) multicentrics (those chromosomes possessing three or more centromeres; for quantification, the number of centromeres present in the polycentric chromosomes minus one was used and scored as dicentric equivalents) (3) centric rings; (4) interstitial fragments, (i.e., chromosome elements without telomeric signals) which includes typical acentric fragments – where chromatids lie parallel throughout their length and there is no centromeric constriction – and double minute chromosomes of variable size; (5) incomplete chromosome elements, both centric (incomplete chromosomes, lacking telomeric signals at one or both ends) or acentric (terminal fragments), (6) compound fragments (acentric fragments labeled at both ends, i.e., exhibiting four telomeric signals), and (7) mono- and isochromatid breaks. The discrimination between centric rings and interstitial fragments (considered by some authors as "acentric rings" [1–3]), was based on the morphological appearance and the presence of an accompanying compound fragment. Besides the above mentioned aberrations, we scored those aberrations directly involving terminal telomeric sequences, i.e., implying telomere dysfunction, as follows: telomere fusions (giving rise to dicentric or ring chromosomes without accompanying fragment), telomere associations, telomeric repeats translocations, additional telomeric FISH signals (which implies translocation and/or amplification of telomeric repeats), extra-chromosomal telomeric FISH signals, and telomere (FISH signal) loss or duplications [2,3]. For the discrimination and scoring of telomere fusions and associations, we used the criteria

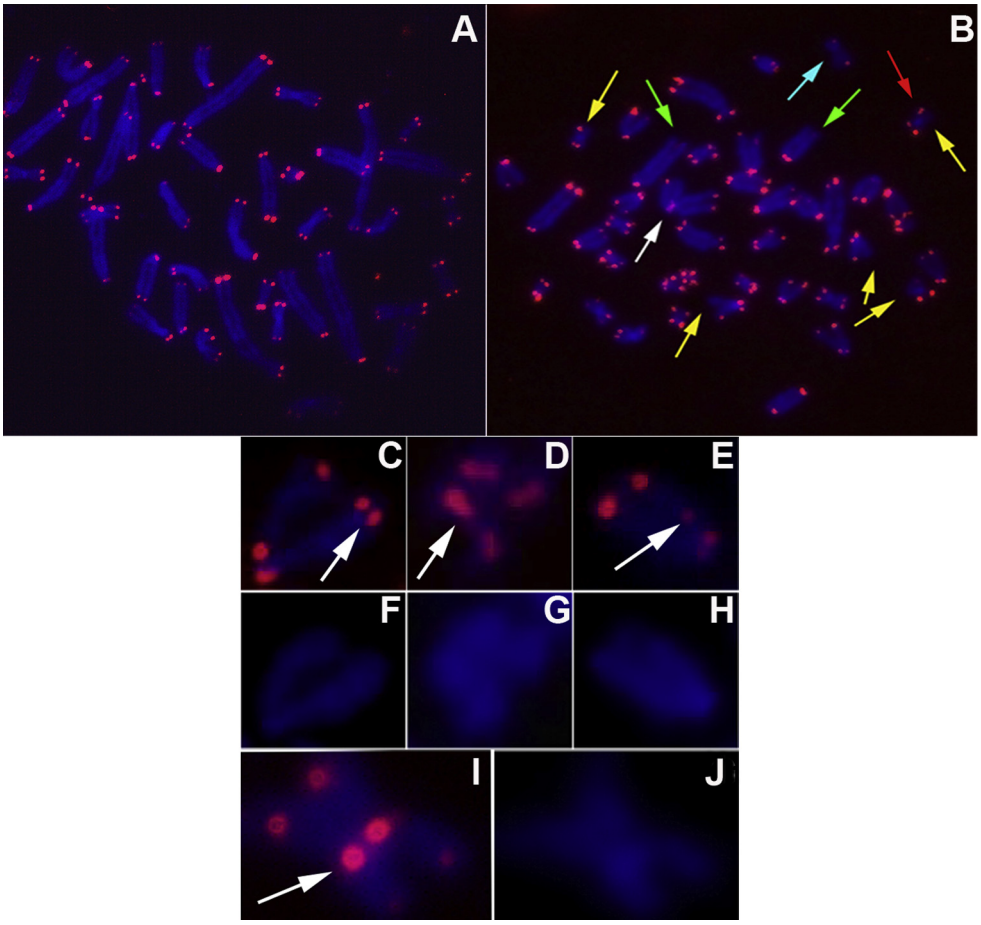

Fig. 1. Patterns of hybridization of (TTAGGG)n repeats in the metaphase spreads from untreated (A) and SN-treated (B-J) ADIPO-P2 cells after PNA-FISH with a pantelomeric probe labeled with Cy3 (red signal). (A) Control metaphase ($2n = 42$) cell. Note the presence of terminal telomeric FISH signals and the absence of interstitial telomeric signals in ADIPO-P2 chromosomes; (B) Full metaphase of a SN-treated culture of ADIPO-P2 cells 10 days after treatment exhibiting different types of telomere aberrations: Five chromosomes with chromatid-type telomere loss (yellow arrows indicate the site where telomere loss occurred), acrocentric chromosomes with chromosome-type telomere loss (green arrows), one acrocentric chromosome with additional telomeric signals (red arrow) at one of its chromatids, a metacentric chromosome exhibiting telomere loss at each opposite chromosome arm (light blue arrow), and one association between two acrocentric chromosomes (white arrow). (C–J) Individual chromosomes from metaphase cells of SN-treated cultures exhibiting different types of telomere aberrations: (C) Acrocentric chromosome showing chromatid-type telomere duplication (arrow). (D) Metacentric chromosome showing telomeric signals duplicated in both arms (the arrow indicates one of the sites of telomere signal duplication). (E) Additional telomeric signals (arrow) at one of the chromatids of an acrocentric chromosome. (F–H, same chromosomes as in (C)–(E), respectively, as seen with DAPI filter). (I) Telomeric fusion between two acentric chromosomes (arrow shows the corresponding two telomeric signals). (J) Same aberration as in (I) seen with DAPI filter.

indicated in refs. [2] and [3]. Thus, telomere associations are aberrations implying that the telomeres of two different chromosomes are very close one to each other. Therefore, we scored as telomere associations those aberrations recognized by the presence of two pairs of very close telomeric signals after PNA-FISH, each pair of signals corresponding to a different chromosome. On the contrary, telomere fusions imply the fusion of two chromosome ends, so we scored as telomere fusions those aberrations where the telomeres of adjoining chromosomes have fused into a single telomere FISH signal, one per chromatid, and the DAPI signal was continuous through the point of fusion.

2.4. Telomerase activity assay

Telomerase activity was measured in all samples by using the TRAPeze-RT telomerase detection Kit (Millipore, Bedford, MA, USA) and Taq Platinum (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. 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. Briefly, after the indicated treatment, cell extracts were resuspended in 100 μl of CHAPS lysis buffer. Protein levels were evaluated using the Quant-iT Protein Assay Kit with the Qubit Fluorometer (Invitrogen, Grand Island, NY, USA). Extracts containing 1.5 μg/μl proteins were used for telomerase assays. A standard curve was generated, based upon measurement of the reactions performed with TRS8 (control template). Positive and negative controls were examined accordingly. 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. Assays were performed on a Real time fluorescence capable thermocycler with FAM detection ABI Prism 7500 (Bio-Rad Laboratories Inc., Irvine, CA, USA). Results were expressed as percentage change of telomerase activity in SN-treated cells compared with untreated cells, following Fridlender et al. [20] and our previous work with bleomycin [12].

2.5. Statistical analysis of data

Statistical analysis of data was performed using GraphPad Prism version 4.00 software for Windows (GraphPad Software, San Diego, CA, USA). Comparisons between control vs. exposed cultures in the percentage of damaged cells were carried out using

Table 1a
Chromosomal aberrations not involving telomere dysfunction observed in untreated and SN-exposed ADIPO-P2 cells after PNA-telomere FISH scoring.

Treatment	Number of cells analyzed	Dic	Rings	IC[a]	Acentric fragments	Breaks		Total aberrations (frequency/cell)
						Chromatid type	Chromosome type	
Control (18 h)	181	4	0	18	18	7	3	50(0.28)
Control (10 d)	199	2	0	22	27	4	2	57(0.29)
Control (15 d)	143	2	0	8	10	1	1	22(0.15)
SN 100 ng/ml (18 h)	134	2	2	10	14	2	4	34(0.25)
SN 100 ng/ml (10 d)	157	14	2	13	17	1	5	52(0.33)
SN 100 ng/ml (15 d)	128	4	2	14	17	7	2	46 (0.36)*

SN, streptonigrin; IC, incomplete chromosomes (chromosomes lacking one end and accompanied by an acentric fragment).
[a] Those chromosomes lacking telomere FISH signals at one or both of their ends but without accompanying acentric fragment were considered as events of chromosome-type telomere loss (included in Table 1b) instead of IC.
* $p < 0.05$ compared with the corresponding control value.

the Chi-squared 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 [21]. The Paired *t* test was used to determine the statistical significance of the differences in telomerase activity between SN-treated and control cells. ANOVA with the Kruskal–Wallis test was used to determine the statistical significance of the differences in telomerase activity between the different SN-treated samples. Differences were considered to be statistically significant at two-sided *p* values < 0.05.

3. Results

3.1. Chromosome damage and distribution pattern of telomeric sequences in untreated cells

As expected from previous studies in our laboratory [12], ADIPO-P2 cells showed telomeric repeats localized exclusively at the terminal regions of chromosomes, some FISH signals being stronger than others (Fig. 1A).

On the other hand, the high spontaneous frequency of incomplete chromosomes and acentric fragments observed in control cells (Table 1a), cannot be linked to the results of the FISH analysis and is similar to the one found in other mammalian cell lines previously investigated in our laboratory for telomere instability [18,22–24]. Furthermore, the significant increase in the frequency of telomere FISH signal loss found in control cells 10 and 15 days after treatment could be due to spontaneous extensive telomere shortening, a phenomenon usually observed in *in vitro* cultured cells [1–4].

3.2. Persistence of chromosome damage induced by SN

SN induced a significant increase in the percentage of aberrant metaphases (i.e., cells showing at least one aberration) (Fig. 2), as well as in the frequency of total chromosomal aberrations per cell (Fig. 3; see data on Tables 1a and 1b) in ADIPO-P2 cells at 18 h and 10 and 15 days after treatment ($p < 0.05$). This shows that the chromosome damage induced by SN remains stable at least up to 15 days after treatment. The fact that we could not obtain enough amounts of metaphases for aberration scoring in SN-treated cultures as well as in control cells, suggests that some cells die because of severe chromosome damage, indicating that probably a selection force against these damaged cells could have occurred. However, our results show that even if the above mentioned event exists, it is not significant enough to have an effect on cell viability (refer to the end of this section).

A detailed analysis of data showed that significant induction of chromosomal aberrations by SN was mostly observed for those aberrations related to telomere dysfunction (Table 1b and Fig. 4). In

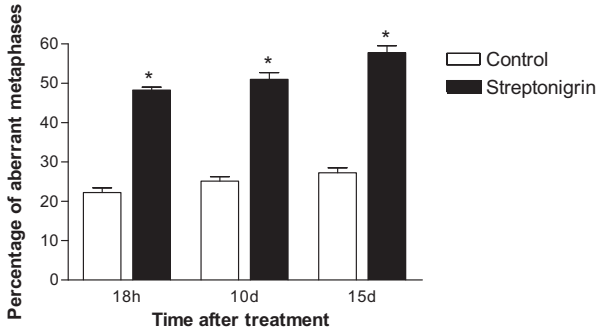

Fig. 2. Percentage of aberrant metaphases (i.e., cells showing at least one chromosomal aberration) in continually subcultured (proliferating) ADIPO-P2 cells as a function of time (h = hours; d = days) after treatment with SN (100 ng/ml). Data represent average values from two independent experiments. For each treatment, mean ±S.E. is indicated. Chi-squared test indicated significant differences (*) between control and exposed cultures at all the time points analyzed ($p < 0.05$).

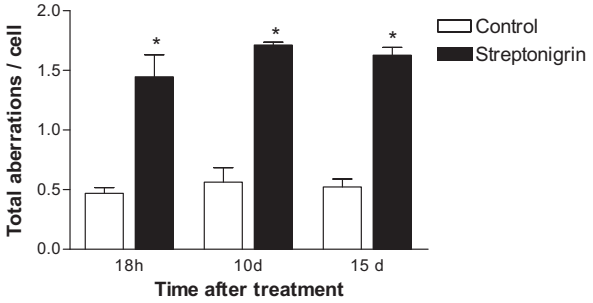

Fig. 3. Changes in the frequency of chromosomal aberrations (all aberrations included; see data in Tables 1a and 1b) in continually subcultured (proliferating) ADIPO-P2 cells as a function of time (h = hours; d = days) after treatment with SN (100 ng/ml). Data represent average values from two independent experiments. For each treatment, mean ±S.E. is indicated. The Z score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by SN at all the time points analyzed compared with untreated (control) cultures ($p < 0.05$). For this analysis, dicentrics and centric rings involving telomere fusion were counted as one aberration each.

effect, induction of aberrations not implying telomere dysfunction was only detected in metaphase cells derived from cell cultures harvested 15 days after SN treatment (Table 1a). Moreover, no induction of incomplete chromosomes and acentric fragments by SN was observed in ADIPO-P2 cells (Table 1a).

Table 1b shows that SN induced telomere dysfunction in ADIPO-P2 cells at 18 h and 10 and 15 days after treatment ($p < 0.05$, Fig. 4), cytogenetically detectable mainly as additional telomeric FISH signals, extra-chromosomal telomeric FISH signals, chromatid- and chromosome-type telomere FISH signal loss, and chromatid-type telomere FISH signal duplication (Table 1b; Fig. 1B–I). The yield of

Table 1b
Chromosomal aberrations implying telomere dysfunction observed in untreated and SN-exposed ADIPO-P2 cells after PNA-telomere FISH scoring.

Treatment	Number of cells analyzed	Telomere fusion	Telomere association	Translocation of telomeric sequences	ATS + ECTS	Telomere loss (chromosome/ chromatid)	Telomere duplication (chromosome/ chromatid)	Total aberrations (frequency/cell)
Control 18 h	181	0	4	2	2	14/5 (19)	2/5 (7)	34 (0.19)
Control 10 d	199	1	0	0	0	18/23 (41)	2/11 (13)	55 (0.28)
Control 15 d	143	2	0	1	4	20/26 (46)	1/1 (2)	55 (0.38)
SN 100 ng/ml 18 h	134	2	0	5	14	54/62 (116)	5/20 (25)	162 (1.21)*
SN 100 ng/ml 10 d	157	8	2	3	17	68/86 (154)	5/36 (41)	225 (1.43)*
SN 100 ng/ml 15 d	128	3	4	3	8	61/36 (97)	6/44 (50)	165 (1.29)*

SN, streptonigrin; ATS, additional telomeric FISH signals; ECTS, extra-chromosomal telomeric FISH signals. Telomere fusions include both dicentrics and centric rings involving fusion of their ends (these aberrations were also included in Table 1a). In the case of telomere loss and duplication, the number of chromosome- and chromatid-type telomere loss and duplications and the total of these aberrations (in brackets) are indicated.
* $p < 0.05$ compared with the corresponding control value.

SN-induced aberrations remained very similar at 18 h, 10 days as well as 15 days after treatment (Fig. 4 and Table 1b). In addition, our results show that SN induced 1.9–4.6 times more telomere FISH signal loss than duplication in ADIPO-P2 cells, this effect depending on the time point analyzed (Table 1b).

Analysis of the number of live cells in each subculture or passage showed that the number of live cells per ml of culture medium was similar for control and SN-exposed cells at every time points investigated (Fig. 5). Thus, there was no SN-induced delayed cell death in ADIPO-P2 cells. The non-significant trend in cell mortality associated with the 10 days harvesting time could be ascribed to technical variations in the method used for cell count, since this effect was observed in both untreated and SN-treated cells.

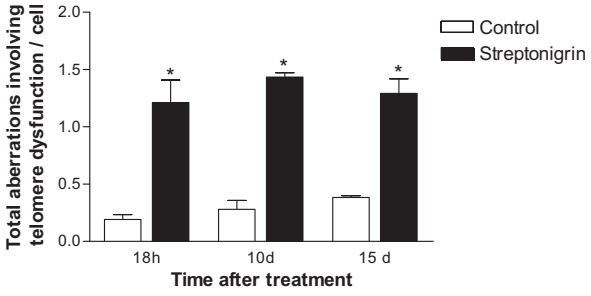

Fig. 4. Changes in the frequency of chromosomal aberrations implying telomere dysfunction (see data in Table 1b) in continually subcultured (proliferating) ADIPO-P2 cells as a function of time (h = hours; d = days) after treatment with SN (100 ng/ml). Data represent average values from two independent experiments. For each treatment, mean ±S.E. is indicated. The Z score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by SN at all the time points analyzed compared with untreated (control) cultures ($p < 0.05$).

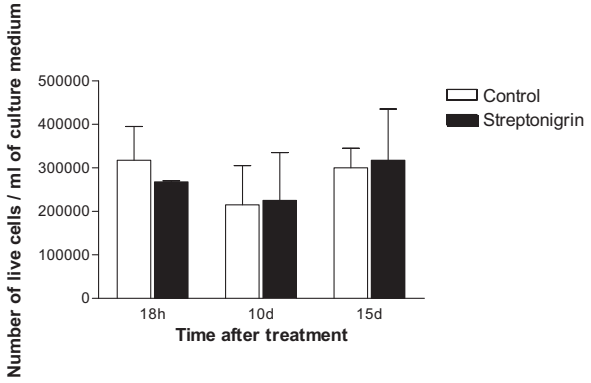

Fig. 5. Variation in the number of live cells observed in ADIPO-P2 cells with time in culture after treatment with SN (100 ng/ml). Data represent average values from two independent experiments. For each treatment, mean ±S.E. is indicated. No significant differences were found between treatments.

3.3. Telomerase activity in SN-treated and untreated cells

Fig. 6 shows the level of telomerase activity in SN-treated cells compared with untreated control cells observed at every time points analyzed. Telomerase activity decreased in SN-treated cells compared with untreated control cells, by a mean of 54, 77 and 74% at 18 h and 10 and 15 days after treatment, respectively ($p < 0.05$) (Fig. 6). Moreover, statistical analysis of data showed that the level of telomerase activity in SN-treated cells did not differ between the different time points analyzed ($p > 0.05$), indicating that the decrease in telomerase activity does not depend on the elapsed time after treatment.

4. Discussion

The study of the long-term effects of antineoplastic drugs on telomeres is fundamental to understand the genomic instability associated with chemotherapy regimens, since telomere loss or attrition seems to play a key role in this instability. Several studies show that cancer patients undergoing chemotherapy have shorter telomeres in their blood cells (even after the end of treatment) [25–30]. A recent report supporting this view provides evidence *in vivo* that telomere dysfunction promotes carcinogenesis in mice [31]. Furthermore, another recent study showed that the chemotherapeutic agents doxorubicin and etoposide as well as γ-radiation induce short-term telomere dysfunction in normal human T lymphocytes and fibroblasts. Consequently, these cells exhibited telomere shortening, down-regulation of telomerase activity, and diminished expression of telomerase reverse transcriptase (hTERT) and the telomere binding proteins TPP1 and POT1 [32]. This suggests that repeated exposure to chemo- and radiotherapy may cause telomere dysfunction [32].

In our previous work, we explored the long-term effects of the chemotherapeutic agent bleomycin on telomeres of rat cells [12]. In the present study, we investigated if SN also induces telomere instability in the progeny of mammalian cells several generations after exposure. Additionally, we investigated the long-term clastogenic effect of SN in terms of unstable chromosomal aberrations not involving telomere dysfunction. Although we found that SN induced chromosome damage in ADIPO-P2 cells at 18 h as well as 10 and 15 days after treatment, significant induction of chromosomal aberrations by this compound was mostly observed for those aberrations related to telomere dysfunction. Induction of aberrations not implying telomere dysfunction was only detected in metaphase cells derived from cell cultures harvested 15 days after SN treatment. In contrast, previous studies in human lymphocytes and Chinese hamster cells showed that SN induces chromosome

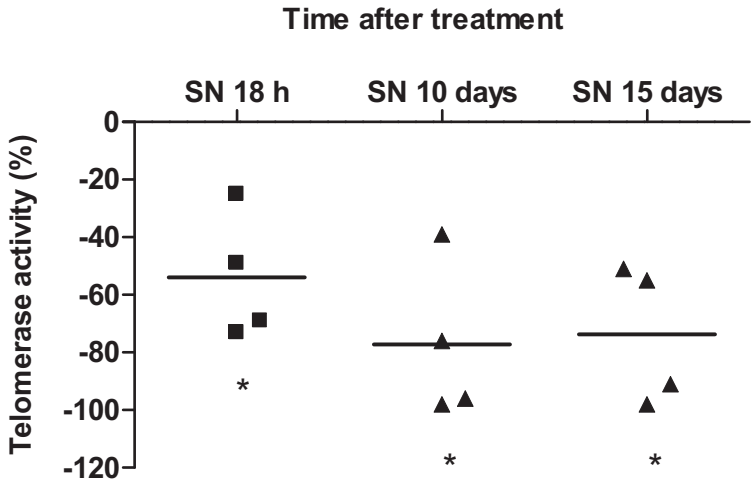

Fig. 6. Percentage change of telomerase activity in SN-treated compared with untreated ADIPO-P2 cells at 18 h and 10 and 15 days after treatment. Telomerase activity was assessed using the TRAPeze-RT Telomerase Detection Kit. Each point represents an independent comparison between SN-treated and control cells. Kruskal–Wallis test showed no significant differences between SN-treated samples. (*) Denotes statistically significant differences between control and SN-treated samples ($p < 0.05$).

damage since the first mitosis after treatment [11,15–19]. Moreover, we previously found that in Chinese hamster embryo (CHE) cells SN induces incomplete chromosome elements (i.e., chromosome end loss), and that incomplete chromosomes and terminal fragments are the most frequent asymmetrical chromosomal aberrations induced by this compound in these cells at 18 h after treatment [18]. Overall, these data indicate that mammalian cells exhibit differential sensitivity to SN clastogenic action, and that this sensitivity depends on the type of aberration considered. The reason why this happens remains to be determined and requires further experiments with the ADIPO-P2 cell line in order to be elucidated.

Our present results show that SN induces telomere dysfunction in ADIPO-P2 cells in the form of additional telomeric FISH signals, extra-chromosomal telomeric FISH signals, and telomere FISH signal loss and duplications. Telomere FISH signal loss and duplication were also observed in X-ray-surviving human fibroblasts 14 days after exposure [5] and bleomycin-exposed ADIPO-P2 cells 10 days after treatment [12]. Therefore, these aberrations, directly related to telomere dysfunction [1–3], seem to be a signature of telomere instability induced by radiation and radiomimetic agents. Since most of SN-induced telomere duplication events were of chromatid-type, we may assume that these aberrations mainly occur during DNA replication or in the G2 phase of the cell cycle. In addition, the induction of telomere FISH signal loss by SN seems to occur throughout the cell cycle, since this compound produced about the same frequency of chromosome- and chromatid-type telomere loss. On the contrary, bleomycin [10] and X-ray-induced telomere instability [5] seems to occur during DNA replication or in the G2 phase of the cell cycle, as most of the induced aberrations were of chromatid-type.

SN-induced telomere dysfunction was observed in ADIPO-P2 cells harvested 18 h and 10 and 15 days after treatment, so we can conclude that SN induces persistent telomere dysfunction in mammalian cells. According to our data, SN-induced telomere dysfunction observed at 10 and 15 days after treatment is due to derived aberrations scored after the first cell cycle after treatment, since the same telomere-related aberrations were also present in cells harvested 18 h after treatment. Similar data was previously obtained with bleomycin-exposed ADIPO-P2 cells [12]. These results differ from the findings by Ojima et al. [5] that X-rays induce delayed telomere instability (in the form of telomere FISH signal loss and duplication) in human fibroblasts, and the observations by Tanaka et al. [6], who found delayed induction of telomere associations or fusions in human B-cell lines established from α-ray- and γ-ray-irradiated lymphocytes. Thus, while telomeres of cells exposed to ionizing radiation appear to be unstable several generations after exposure, those of radiomimetic compounds-exposed cells are unstable since the first cell division after exposure.

We previously observed in Chinese hamster cells (CHO and CHE cell lines) that telomerase activity in SN-treated cells was similar to that of untreated cells 18 h after treatment [33]. However, our present study shows that ADIPO-P2 cells exposed to SN exhibit significantly lower telomerase activity than untreated cells. This finding suggests that this compound produces telomerase activity inhibition in ADIPO-P2 cells, which persists for at least 15 days after treatment. Thus, the effect of SN on telomerase activity seems to be dependent on the cell type. The discrepancies on telomerase activity between Chinese hamster and ADIPO-P2 cells after being treated with SN, could be due to the methodology used to determine the activity of telomerase, since quantitative (q-PCR) assay (present study) is much more sensitive than polyacrylamide gel electrophoresis analysis (previous study). In the present study, the persistent decrease in telomerase activity in SN-exposed ADIPO-P2 cells was accompanied by the persistence of aberrations directly related to telomere dysfunction, especially telomere FISH signal loss. Therefore, it is valid to assume that telomerase could be involved in SN-induced telomere dysfunction. Thus, telomerase inhibition could be a key event contributing to telomere dysfunction in SN-exposed cells.

The inhibition of telomerase activity by SN could be either a direct or an indirect effect of the drug. SN could directly inhibit telomerase activity by affecting its catalytic reverse transcriptase subunit (known as hTERT) or the RNA subunit of the enzyme (termed hTERC or hTR) [34]. In addition, SN could inhibit telomerase activity by altering some of the proteins associated with telomerase or the telomere (the shelterin complex) or acting on the telomere transcripts termed Telomeric Repeat containing RNA (TERRA) [34]. Our present study does not allow us to determine if SN has a direct effect on the components of telomerase, the proteins associated with this enzyme, the shelterin complex or TERRA. Besides, there is no data available in literature concerning this issue. Alternatively, the decreased telomerase activity in SN-treated cells could be a consequence of cell death induced by the drug. However, our present data suggest that the decreased telomerase activity in SN-treated cells is not related with cell proliferation, since no

significant changes were observed in the number of live cells/ml of culture medium in the cell cultures analyzed. Therefore, further studies will be needed to determine the precise mechanism by which SN inhibits telomerase activity in mammalian cells.

Based on our present findings and previous data, several mechanisms underlying SN-induced toxic effects could be involved in the persistent telomere instability induced by SN in ADIPO-P2 cells. First, SN is a topoisomerase poison, which inhibits topoisomerase II by stabilizing the trans-esterification intermediate of the enzyme (called cleavable complex), inducing DNA double strand breaks [13,35]. Thus, SN could promote telomere cleavage by topoisomerase II, such effect may contribute to telomere shortening in the exposed cells. In effect, there is evidence that topoisomerase II recognizes and cleaves telomeric DNA. Futhermore, many topoisomerase II poisons promote cleavable complex formation at telomeric DNA sequences, producing telomere damage [36]. Moreover, as stated before, etoposide, a well-known topoisomerase poison, induces short-term telomere dysfunction in normal human cells as a consequence of telomere shortening, down-regulation of telomerase activity, and diminished expression of telomerase reverse transcriptase (TERT) and the telomere binding proteins TPP1 and POT1 [32]. This suggests that SN might act on telomeres and telomerase in a similar way to etoposide, due to its topoisomerase II inhibitory effect, thus promoting telomere dysfunction. Second, several studies have provided a large body of evidence indicating that SN directly interacts with DNA, binding covalently but not interacting into the double helix (see [13] for review). Therefore, the persistence of the clastogenic action of SN in terms of telomere-associated aberrations could be due to the formation of a stable complex between SN and the DNA molecule, which may induce chromosome damage through a persistent cyclic redox process and the resulting generation of active oxygen species [17]. Third, as our present data suggest, SN causes inhibition of telomerase activity, an effect also observed in human cells treated with the topoisomerase II poison etoposide [32]. A decreased telomerase activity could promote extensive telomere shortening, cytogenetically detected as telomere FISH signal loss in SN-treated cells. This assumption is based on the fact that the PNA telomeric probe, such as the one used in our present study, is highly specific for the TTAGGG sequence, and the fluorescence intensity of the spots is directly correlated to the length of the telomeric repeats [37,38]. Our data indicate that SN induces about 2–4 times more signal loss than duplication, being telomere loss the most significant effect of SN on telomere function at the chromosome level. It should be noted that since SN is a good inducer of sister-chromatid exchanges (SCEs) [13], it is reasonable to assume that the effect of this compound on telomeres could be partially due to the SCE-inducing activity of SN at the telomeric level (i.e., the so-called T-SCEs,) [1–3]. Aberrations such us additional telomeric FISH signals and extra-chromosomal telomeric FISH signals could result from recombination events at telomeres. However, no data are available yet regarding the induction of T-SCEs by SN, so further studies are needed to elucidate this issue.

Previously, we found that bleomycin, another radiomimetic compound, induces telomere instability in mammalian cells (cytogenetically detectable as incomplete chromosome elements and telomere FISH signal loss and duplication) which persists for at least 10 days after treatment [12]. Thus, both SN and bleomycin induce persistent telomere instability in mammalian cells. Although SN and bleomycin are radiomimetic compounds, they exhibit some important differences in their long-term effects on telomeres (see [12] and present work), indicating that chemotherapeutic drugs differ on their effects on telomeres. Firstly, bleomycin induces persistent chromosome end loss and telomere dysfunction, whereas SN induces persistent telomere dysfunction. Secondly, bleomycin induces telomere fusions, whilst chromosome end loss and telomere fusions were not detected in SN-treated cells. Finally, bleomycin induces delayed increase of telomerase activity in mammalian cells, while SN decreases telomerase activity. Although these discrepancies could be due to differences between SN and bleomycin in their mode of action [13,39,40], further studies will be needed to confirm this assumption.

In summary, our present work shows that the antitumor antibiotic SN induces persistent telomere instability in mammalian cells, cytogenetically manifested as telomere dysfunction-related chromosomal aberrations. Therefore, our present study suggests that one of the mechanisms by which SN causes long-term genomic instability is telomere dysfunction. Alternatively, the observed telomere instability by SN could be partially due to the induction of telomere sister-chromatid exchanges (T-SCEs) by this compound. Further studies should be aimed at confirming our present assumptions on the mechanisms underlying the long-term induction of telomere instability by SN in mammalian cells, particularly whether telomerase plays a significant role in this instability. In this case, it would be interesting to determine if telomerase overexpression protects against SN genotoxic effects. These studies will help to better understand the effects of radiomimetic antitumor antibiotics on telomeres. Moreover, since the chromosomal sensitivity of mammalian cells to SN and the effect of this compound on telomerase activity differ between cell types, it will be of great interest to perform similar experiments in other cell types to allow more general conclusions on SN-induced telomere instability.

Conflict of interest

None.

Acknowledgments

This work was supported by grants from CONICET (PIP No. 0316) and CICPBA of Argentina. We wish to thank Julieta Parisi for technical assistance, and Dr. Jorge López Camelo for assistance with statistical analysis of data.

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