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ORIGINAL ARTICLE

Deregulated sequential motion of centromeres induced by antitumor agents may lead to genome instability in human peripheral blood lymphocytes

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Summary

Purpose: *Segregation of chromosomes in anaphase is preceded by a sequential order of centromere separation. Alteration of the sequence of centromere separation or premature centromere division (PCD) has been found to be significantly higher in populations exposed to various xenobiotics. The purpose of this study was to investigate if PCD induced by various cytostatics can alter the stability of chromosomes and lead to aneuploidy.*

Materials and methods: *Peripheral blood lymphocytes of 10 healthy, non smoking subjects were exposed to 8-Cl-cAMP at a dose of 1, 5 and 15 μM, paclitaxel at a dose of 0.01, 0.05 and 0.2 μM, and cycloheximide (CX) at a dose of 5, 10 and 25μg/ml. By using the cytohalasin B (CB)-micronucleus (MN) test in vitro, in combination with fluorescent in situ hybridization (FISH), the presence of MN was analyzed in 1000 binuclear cells for each experimental and negative control group. For analysis of MN content we used the α-centromeric probe for chromosome 18.*

Results: *8-Cl-cAMP and paclitaxel induced an increase in the frequency of MN in peripheral blood lymphocytes. 8-Cl-cAMP and paclitaxel proved clastogenic, i.e. they increased the frequency of MN and induced PCD in all respective doses. CX proved no clastogenic in the respected doses when using the CB-MN test in vitro, although CX is a specific PCD inducer. No correlation of PCD and aneuploidy of chromosome 18 was found in cells exposed to 8-Cl-cAMP and paclitaxel by using FISH. In cells exposed to CX we found PCD of chromosome 18 in binuclear cells and single signals in scarce MN. These findings were not statistically significant compared to the negative control group.*

Conclusion: *Our results show that the properties of the investigated antitumor agents to induce PCD in peripheral blood lymphocytes and, therefore, aneuploidy and genome instability, is highly based on the nature of the alteration of centromere function, i.e. the temporal order of centromere kinetics are more regulated through the sequence of centromere separation than by the segregation processes. We suggest that PCD induced by novel antitumor agents could be included in preclinical and clinical genetic risk assessment analysis.*

Key words: aneuploidy, 8-Cl-cAMP, fluorescent in situ hybridization, paclitaxel, premature centromere division

Introduction

In a four-dimensional cascade of cyclin-regulated cell cycle kinetics, chromosomes possess an essential role in maintaining the stability of the fluctuating genome. Human chromosomes are nonrandomly organized in space, and more recent data show a temporal organization of the replication, separation and segregation processes of the chromosomes [1]. Segregation of the chromosomes in anaphase is preceded by a sequential order of separation. Centromere separation is a genetically controlled process in which certain chromosomes are first to separate. In humans the first chromosome to separate, thus segregate, is chromosome 18.

In an ordered sequence, chromosomes 17, 2, 10 and 12 follow the separation of chromosome 18 [2-4]. Those separating last are chromosomes 21, 22, 13, 14

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and 15. This sequence can be deregulated in ageing cells, Alzheimer's disease patients, various tumors, chromosome instability (CIN) syndromes, and by chemical induction [5-11].

Antitumor agents, as well as various xenobiotics, can induce PCD in human peripheral blood lymphocytes [12-14].Treatment of *in vitro* cultured mouse bone marrow cells with alkaloid pyrolizidine has induced PCD and increased the frequency of chromosomal aberrations (CA) [15]. Mouse oocytes exposed to a variety of xenobiotics, including paclitaxel, 1,2 propanediol expressed spindle defects, PCD and aneuploidy [16,17].

PCD was also found in human peripheral blood lymphocytes *in vitro* when treated with a mixture of 15 commonly used pesticides [18]. CIN has been recognized as a hallmark of human cancer caused by continuous chromosome missegregation during mitosis in which errors of centromere separation, particularly premature separation or PCD, are seen to have a fundamental role [19-22].

Evidence is accumulating that CIN plays an important role in the development and progression of cancer because aneuploidy is found in the earliest stages of tumorigenesis, i.e. carcinomas *in situ* or precancerous lesions of many tumor types [23,24]. Aberrant expression and localization of kinetochore proteins CENP-A, CENP-H and centrosome-associated kinase aurora 2 are amplified in human cancers, thus playing an important role in the process of aneuploidy induction [25-28]. Also, exogenous expression of the kinase aurora 2 in rodents and human cells induced unequal partitioning of chromosomes during mitosis and tumorigenic transformation of cells [25-28].

To increase our understanding about the potential risks of chemically-induced aneuploidy, particularly PCD-related aneuploidy, as PCD can be induced by different chemical mechanisms, we used the CB-MN assay as an endpoint for clastogenic, and/or aneugenic action of 8-Cl-cAMP, paclitaxel and CX. Wuttke et al. [29] suggested that cytogenetically damaged chromosomes are not always distributed randomly, but some chromosomes are involved more frequently than others in the formation of MN. PCD induces chromosome instability.

To detect any correlation between PCD of chromosome 18 in mononuclear and binuclear cells, and the aneugenicity of the investigated agents we used the FISH method. Previous studies showed that our investigated agents can induce PCD and aneuploidy in the chromosome aberration assay *in vitro* [13,14,16].

The usage of the MN test and FISH enables us to detect whole chromosomes in the second division of cells exposed to cytostatics. The second division manifests as a binuclear cell with MN. The MN content is analyzed by using FISH for chromosome 18. The α-centromeric region probe for chromosome 18 is basically used because chromosome 18 is the first to separate in the anaphase-metaphase transition.

Alteration of the centromere separation or premature separation of centromeres in metaphase may have consequences on the chromosome stability that can lead to aneuploidy.

Materials and methods

Experimental groups

Two distinctive groups were established for the MN test. One control group or PBS-treated group and one experimental group based on dosage. Paclitaxel was supplemented to the cultures at a dose of 0.01, 0.05 and 0.2 μM; 8-Cl-cAMP at a dose 0.1, 0.5 and 15 μM; and CX at a dose of 5, 10 and 25 μg/ml.

After 24h air drying, cells were stained with 2% Giemsa for 3 min. For MN analysis, at least 1000 binuclear cells were analyzed per culture, registering MN according to the criteria of Fenech [30]. The ability of cells to proliferate *in vitro* was evaluated by counting the number of cells with 1, 2 and 4 MN at the same slide using the nuclear division index (NDI).

Blood samples, culture condition

Peripheral blood lymphocytes from 10 healthy male nonsmokers, aged 25-35 years, were taken in previously heparinized test tubes. Lymphocyte rich plasma was cultured in a density of 5×10^5 cells/ml in RPMI 1640 medium (Gibco, Gr, Isl, NY, USA) supplemented with 10% of heat-inactivated fetal calf serum (Gibco, Eggenstein, Germany), antibiotics (1% streptomycin/penicillin solution). The lymphocytes were stimulated with a growth factor, i.e. phytohemagglutinin (Murex Diagnostics Ltd., Dartford, England) in concentration of 2% for 72 h at 37°C.

Micronucleus assay analysis, treatment

For the *in vitro* CB-MN assay 6 μg/ml CB (Sigma Chemical Co., St. Louis, MO, USA; CAS No 14930-96-2) were added to the cultures 24h before harvesting, according to the Fenech method [30].

All the investigated substances [8-Cl-cAMP (Sigma Chemical Co., St. Louis, MO, USA; CAS No 41941-56-4) at doses of 1, 5 and 15 μM; paclitaxel

(Sigma Chemical Co., St. Louis, MO, USA; CAS No 33069-62-4), CX at doses of 5, 10 and 25 μg/ml (Sigma Chemical Co., St. Louis, MO, USA; CAS No 66-81-9)] were added 48h after the incubation and stayed in culture to the end of cultivation (72 h).

Lymphocytes were centrifuged at 80-100 g for 6-8 min after the incubation period of 72 h. The pelleted cells were treated to preserve the cytoplasm by gently resuspending the cytoplasm in a 5 ml hypotonic solution (0.56% KCl plus 0.9% NaCl mixed in equal volumes) , then resuspending twice in 5 ml cold Carnooy's fixative (methanol/ glacial acetic acid 3: 1 v/v) for 20 min at room temperature. After the final centrifugation at 1000 g for 8 min cells were thoroughly mixed using Pasteur pipette and were dropped from a height of 3-4 cm onto wet, clean slides. For each culture at least two slides were prepared. Slides were air-dried and stained with 2% Giemsa (Merk, Darmstadt, Germany) solution in Gurr buffer (pH 6.8).

Blood samples were coded with an identification number and slides were scored blind. For MN analysis, at least 1000 binuclear cells per culture were analyzed, registering MN according to the criteria of Fenech [30].

The ability of cells to proliferate *in vitro* was evaluated by counting cells with 1, 2 and 4 MN on the same slide using NDI which was calculated according to the following formula:

$$NDI = (M_I + 2M_{II} + 3M_{III} + 4M_{IV}) / N$$

where M_I to M_{IV} represent the number of cells with 1-4 nuclei, respectively, and N is the number of the cells scored.

Application of fluorescence in situ hybridization for analysis of aneuploidy of chromosome 18

Probe preparation

Chromosomes were prepared in the same way as in the experiment for CB-MN assay. The pL1.84 α-repetitive DNA probe for the centromeric region of chromosome 18 was used. The probe was labeled with biotin-16dUTP nucleoside-triphosphate in nick-translation reaction. For each slide approximately 50 ng of probe were mixed in 16 μl of hybridization buffer, consisting of 50% formamide, 10% dextran-sulfate, 1% sodium dodecyl sulfate (SDS), 1x Denhardt's, 2x sodium chlorine and sodium citrate (SSC) (1xSSC:0.15 M NaCl , 0.15 M sodium citrate, pH 7.0).

Hybridization and detection

Hybridization and detection were performed as previously described [31]. The biotinylated probe was detected with avidin-DCS conjugate to yellow-green fluorophore FITC (fluorescein isothiocyanate; exc. max. 523 nm; 4 mg/ml) and biotinylated anti-avidin-DCS (4 mg/ml, Vector Laboratories).

The signals were amplified using 3 layers of avidin FITC. The slides were mounted in 0.4 mg/ml 4'-6-diamino-2-phenyllindole (DAPI) and propidium iodide counterstain in vectashield antifade buffer.

An Olympus RFL BH2 fluorescence microscope with appropriate filter combination for detecting fluorescein and propidium iodide (0-515 nm) was used for signal detection and evaluation. The slides were photographed using Fuji 100 color film.

Statistical analysis

The chi-square test was used for NDI. Statistical analyses for MN frequencies and the percentage of binucleated cells were performed using the Mann-Whitney U-test. A p-value of < 0.05 was considered as indicative of statistical significance for all tests used.

Results

Cycloheximide

Results from the MN test showed that CX did not increase in a statistically significant manner the frequency of binuclear cells with MN between the experimental groups of 5, 10 and 25 μg/ml when compared to the control group.

NDI shows the proliferative capability of peripheral blood lymphocytes when exposed to various doses of the investigated agents. In the experimental groups of CX NDI showed a decrease (1.099, 1.075, 1.062). Using x^2 test, the proliferation index showed high statistical difference ($p<0.001$) between the experimental groups and the control group (PBS). The NDI displayed an antiproliferative action of CX in peripheral blood lymphocytes (Table 1). Also, CX-induced PCD could be detected by FISH in mononuclear and binuclear cells (Figure 1). No FISH signals were detected in scarce MN found.

8-Cl-cAMP

MN test revealed a statistically significant difference regarding the frequency of binuclear cells with MN between the experimental groups of 5 and 15 μM ($p <0.05$); however, only the highest dose of 8-Cl-cAMP (15 μM) induced a highly statistical significant

Table 1. Changes in the number of micronuclei (MN) in peripheral blood binuclear (BN) lymphocytes in the control and experimental groups by increasing doses of cycloheximide (CX), 8-Cl-cAMP(8-Cl) and paclitaxel (TX)

Concentration (μg/ml and μM)	*Number of mono and BN cells*	*Percentage of BN cell*	*Number of MN in 10^3 BN cells*		*NDI[§] (1xM1+2xM2+3xM3+4xM4) × 10^{-3}*
			X	*SD*	
Control (K)	1028	55.7	5	3.03	1.707
5 μg/ml/CX (A)	1014	9	5.2	3	1.099***
10 μg/ml/CX (B)	1063	6.2	5.3	3	1.075***
25 μg/ml/CX (C)	1000	6.1	6.5	2.4	1.069***
1 μM/ 8-Cl (D)	1004	7.8	4.9	2.13	1.085***
5 μM/ 8-Cl (E)	1000	7	5.4	2.9	1.077***
15 μM/ 8-Cl (F)	1010	5.7	9.3*	4.32	1.061***
0,01 μM /PX (G)	1024	8.9	12.5	5	1.100***
0,05 μM /PX (H)	1000	5.6	20.3	5.3	1.063***
0,2 μM /PX (I)	1013	4.2	27.8	8.4	1.039***

[§]NDI=nuclear division index

K:(A: B: C), for U>50, p<1, for the NDI we have x^2>1164; p<0.000*** for K:(A,B,C,D,E,F,G,H,I);

K:D; U=50, p<1	D:E; U=44.50, p<0.677
K:E; U=45.5, p<0.73	D:F; U=19, p<0.019*
K:F; U=21, p<0.028*	E:F; U=22.5, p<0.037*
K:G; U=9, p<0.0002***	G:H; U=14; p<0.00651**
K:H; U=000, p< 0.000157***	G:I; U=5.5; p<0.00076***
K:I; U=000, p< 0.00015***	I:H; U=24.5; p<0.054*

*p<0.05, ** p<0.01, ***p <0.001 (Mann-Whitney U-test for CB-MN and x^2 test for NDI)

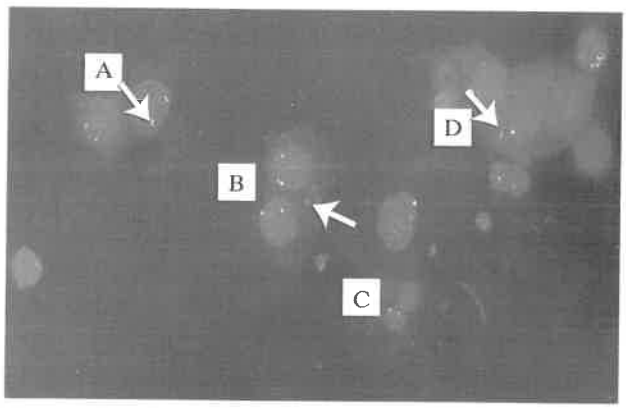


Figure 1. Distribution of FISH signals of chromosome 18 centromeres in binuclear cells induced by cycloheximide at a dose of 10 μg/ml. (A): a binuclear cell with 4 signals in one nucleus (arrow) and 2 signals in the second nucleus. The same distribution of signals can be seen in (B) with the exception of one MN (arrow). Binuclear cells A and B show PCD only where there are 4 signals. In (C) presented is a cell with 2 signals, i.e. without PCD, and in (D) a complex picture with 4 mononuclear cells surrounding one binuclear cell with one rod-like signal (arrow), possibly the beginning of PCD.

difference (p <0.001) compared to the control group. Comparison of the 2 lower dose groups (1 and 5 μM) with the negative control group showed no statistically significant difference (Table 1).

In the experimental groups of 8-Cl-cAMP our results showed a decrease in the NDI (1.085, 1.077, 1.061). Using x^2 test, the proliferation index did not show statistically significant difference between the experimental groups, but between all experimental groups and the control group (PBS) the NDI showed a highly significant difference (p<0.001) and antiproliferative action of 8-Cl-cAMP in peripheral blood lymphocytes (Table 1).

8-Cl-cAMP induced PCD in mononuclear and binuclear cells detected by FISH. No detection of FISH signal in MN was detected (Figure 2).

Paclitaxel

MN test showed a highly statistically significant difference regarding the frequency of binuclear cells

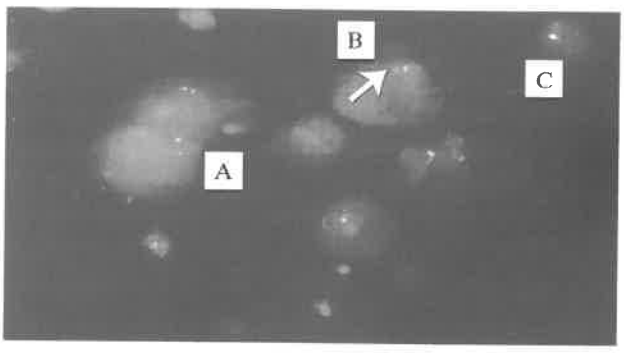


Figure 2. Distribution of FISH signals of the chromosome 18 centromeres in binuclear and mononuclear cells induced by 8-Cl-cAMP at a dose of 5 μM. (A): a binuclear cell with 2 signals in both nuclei with one MN indicating that PCD has not been induced; in (B) a binuclear cell with 2 signals; one signal is very faint (arrow); in (C) 2 mononuclear cells with one signal. This picture is to show how signals are distributed when PCD of chromosome 18 is not present.

with MN between the experimental groups of 0.01, 0.05 and 0.02 μM ($p < 0.01$) compared with the control group (Table 1). Also, the MN test showed a statistically significant difference ($p < 0.01$) regarding the frequency of binuclear cells with MN between the experimental groups of 0.01 and 0.05 μM.

NDI showed proliferative capability for the peripheral blood lymphocytes. In the experimental groups of paclitaxel our results revealed a decrease in the NDI (1.100, 1.063, 1.039). Using x^2 test, the proliferation index showed a highly significant difference ($p<0.001$) between the experimental groups of 0.01 and 0.05 μM. Also, NDI showed highly significant difference ($p<0.001$) between all experimental groups and the control (PBS) group (Table 1). Paclitaxel induced PCD in mononuclear and binuclear cells detected by FISH (Figure 3).

The results showed no correlation between the number of MN, PCD of chromosome 18 and aneuploidy.

Analysis of PCD of chromosome 18 by FISH

Even though a relatively small number of signals were found in MN, showing no statistical significance, some features of the signals in binuclear cells should be addressed:

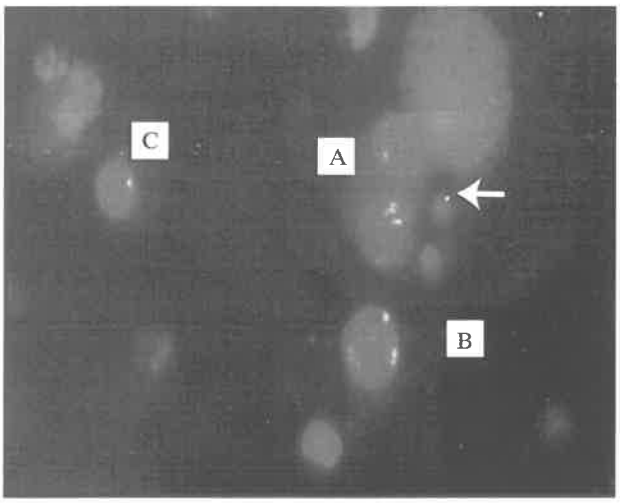


Figure 3. Distribution of FISH signals of the chromosome 18 centromeres in binuclear and mononuclear cells induced by paclitaxel at a dose of 0.05 μM (A): a binuclear cell with one nucleus showing 2 rod-like signals suggesting the end of S and beginning of G2 phase of the cell cycle, which might be an initiation of PCD, and one nucleus with one signal and a signal in one MN (arrow). In (B) a mononuclear cell with one rod-like signal and a dot-like signal. In (C) a mononuclear cell with one dot-like signal suggesting chromosome loss. Again no correlation has been found between chromosome 18 aneuploidy and paclitaxel capacity to induce PCD.

1) Some nuclei in binuclear cells were represented by 4 separate signals of the centromeric region of chromosome 18, indicating complete segregation of chromosome 18 centromeres or PCD (Figure 1B)
2) Some nuclei showed rod-like signals, suggesting the end of the S phase and the beginning of the G2 phase of the cell cycle, which might be the initiation of PCD of chromosome 18 (Figure 1A; Figure 3C)
3) Two separate dot-like signals showed a possible G2 phase of the cell cycle, meaning that no PCD was induced (Figure 2A; Figure 1, 3).

The results taken by FISH clearly revealed the properties of the investigated substances to induce PCD of chromosome 18; they also indicated that PCD of chromosome 18 is found in first (mononuclear cells) and second (binuclear cells) mitosis but not in MN.

Discussion

Increasing evidence in recent years has reaffirmed the aneuploidy hypothesis [21,23]. Aneugenic properties of a number of antitumor agents used in cancer therapy raise concern over the potential risk of developing second cancers after the primary therapy, including germline mutations [32]. PCD is today a well-recognized phenomenon associated with aneuploidy and can be induced by various chemical agents [12,13]. Induction of PCD by chemical agents is twofold; the first alteration is related to the cell cycle *per se* or PCD can be expressed very early, immediately after the G2 phase [4] with the sequence of centromere separation that is not changed [4]. The second way of PCD expression is that no early or interphase PCD detection is apparent, but PCD occurs in the metaphase of the cell cycle expressing also an alteration in the sequence of centromere separation [5,22,33-36]. The latter or the alteration of the sequence induced by chemical agents show a potency to induce aneuploidy. Wuttke et al. [29] investigated the aneugenic potential of some chemical agents and found that there is a non-random distribution of chromosomes in the MN, i.e. some chromosomes are more represented than other chromosomes during the process of MN formation. Chromosome 7 is to be preferentially found in MN induced by colchicine at a dose of 0.02 – 0.8 μg/ml. One explanation is that each chromosome is not attached by the same amount of spindle fibers, i.e. chromosome 7 might contain a relatively low number of microtubules, so that the probability of the chromosome becoming a laggard as the result of colchicine-induced

disruption of the spindle is relatively high [29]. Changes in chromosome 7 are found in various tumors, from glioma to carcinoma of the prostate and the bladder [10,37]. Changes to chromosome 7 are in the vicinity of the c-myc protooncogene [29]. Interestingly 80% of the changes to chromosome 7 are found in secondary tumors occurring after primary cytotoxic therapy [32,37].

One characteristic feature of bladder carcinoma is chromosome 7 instability, expressed as centromere splitting [10]. So, is instability initiated by alteration in centromere dynamics, thus activating the c-myc oncogene or is there a connection between instability, preferential occurrence of chromosomes in MN and aneuploidy subsequently leading to possible carcinogenesis?

Our results do not go in favor to the proposed hypothesis. CX *per se* didn't increase the number of MN in human peripheral blood lymphocytes when compared to the control group, nor gave a FISH signal from the centromere region of chromosome 18. Still, CX is a highly potent inducer of PCD [3,4,13].

Using FISH we didn't observe the existence of signals in MN at all doses of CX, 8-Cl-cAMP and paclitaxel. In the nuclei of BN cells treated with 8-Cl-cAMP there has been no detection of signal for chromosome 18 alpha centromeric region.

Our results show that 8-Cl-cAMP, paclitaxel and CX do not preferentially affect chromosome 18 in the formation of MN, and consequently, aneuploidy of chromosome 18. In Figure 3 only one signal was observed, still no statistical significance has been found that chromosome 18 is preferentially distributed in the MN.

Still, PCD of chromosome 18 has occurred in nuclei of binuclear and mononuclear cells, indicating instability of the cells exposed to the investigated antitumor agents. Also, all these agents showed a capability to decrease the NDI (Table 1) when compared to the control group, indicating toxicity to the mitotic machinery.

In defining the overall toxic potential of PCD induction by various cytostatics we must consider some findings relative for PCD evaluation. Firstly, in the experimental model of PCD Spremo-Potparevic et al. [4] showed that the occurrence of PCD correlates with the normal sequence of centromere separation, i.e. that PCD occurs first on chromosome 18, then on 2, 10, 8, etc and the last chromosomes to separate are the chromosomes of the G and D group in interphase of the cell cycle.

Secondly, Vig et al. [34] showed that a fluorescent dye (HOECHST 33258) generates alterations to the sequence of centromere separation, i.e. chromosomes of D and G group are first to separate (an opposite to CX). Also, cells exposed to the HOECHST dye express aneuploidy. Third, even though no signals have been found in MN, this doesn't mean that other chromosomes are not present in the MN formation. Our results show clearly that genetic instability and possible preferential occurrence of whole chromosomes in MN formation correspond to the alteration of the sequence of centromere separation, not to PCD *per se*.

Susceptibility to PCD and aneuploidy in various syndromes [6,38-42] and the fact that various chemicals with different action toward DNA can induce out-of-phase centromere separation and alter the sequence of centromere separation show a need to define this parameter of genome instability more accurately, therefore our results suggest that PCS induced by novel investigated substances should be included as a parameter of genotoxicity in preclinical and clinical risk assessment analysis.

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