Original Paper

Pathobiology 1994;62:180–185

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Cell Cycle Disturbance in Relation to Micronucleus Formation Induced by the Carcinogenic Estrogen Diethylstilbestrol

Key Words
Flow cytometry
Micronucleus formation
Diethylstilbestrol
Hoechst 33258 dye
Bromodeoxyuridine labeling, continuous

Abstract

In addition to its tumor-promoting activity in hormone-receptive tissue, the carcinogenic estrogen diethylstilbestrol (DES) has been found to induce cell transformation, aneuploidy and micronucleus formation in mammalian cells. The majority of these micronuclei contained whole chromosomes and were formed during mitosis. Here a possible relationship between a disturbance in cell cycle progression and micronucleus formation is investigated by exposing Syrian hamster embryo (SHE) cells to DES. Continuous bromodeoxyuridine labeling followed by bivariate Hoechst 33258/ethidium bromide flow cytometry was employed for analysis of cell cycle transit and related to the time course of micronucleus formation. Treatment of SHE cells with DES resulted in delayed and impaired cell activation (exit from the G0/G1 phase), impaired S-phase transit and, mainly, G2-phase traverse. Cells forming micronuclei, on the other hand, were predominantly in G2 phase during DES treatment. These results suggest that impairment of S and G2 transit may involve a process ultimately leading to micronucleus formation.

Introduction

Certain estrogens like diethylstilbestrol (DES) induce cancer in experimental animals and are associated with tumor formation in man [1]. In addition to the tumor-promoting activity in hormone-receptive tissue, changes at the chromosomal level have been described [2], and numerical chromosome alterations rather than gene mutations have been related to estrogen-induced neoplastic cell transformation [2]. DES has been reported to induce morphological cell transformation, aneuploidy and micronucleus formation in Syrian hamster embryo (SHE) fibroblasts [2, 3]. Using an antibody to kinetochores, it has been shown that the majority of these micronuclei contains whole chromosomes and is formed during mitosis [3]. The mitotic stage of the chromosome cycle is prepared during the G2 phase. In this study we investigated the effect of DES on cell-cycle progression, in particular the G2 phase, in SHE cells and the time course of micronucleus formation in order to assess a possible relationship between these two processes.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 172.

Received:
February 2, 1994
Accepted:
June 8, 1994

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1015–2008/94/0624–0180
$8.00

Materials and Methods

Chemicals

Bromodeoxyuridine (BrdU), DES, Hoechst 33258 (Hoechst) and ethidium bromide were from Sigma Chemical Company (St. Louis, Mo., USA). Dimethylsulfoxide (DMSO) was from Aldrich Company Europe (Nettetal, Germany).

Cell Culture

SHE cells were established as described previously [4]. All experiments were performed with cultures derived from 13-day-old SHEs. Cell cultures were grown in a humidified atmosphere with 12% CO_2 in air at 37°C. The culture medium used was IBR-modified Dulbecco's reinforced medium (Gibco, Karlsruhe, Germany) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 3.7 g/l $NaHCO_3$ and 15% fetal bovine serum (Gibco, Karlsruhe, Germany).

In vitro Micronucleus Assay

SHE cells were plated in 35-mm Petri dishes containing glass coverslips and grown to medium density. Then the culture medium was replaced by a culture medium containing DES (dissolved as a 100 × stock solution in DMSO) or 1% DMSO. Following an incubation period of 5 h the drug was removed by changing the medium. At 12 h (or different time points in time-course experiments) cells were fixed with methanol for at least 30 min at –20°C. The slides were then stained (1 µg/ml Hoechst, 5 min), mounted for microscopy and evaluated for micronucleus formation. Each data point represents the mean of three treated cultures from one experiment with 2,000 nuclei evaluated in each case.

For experiments with synchronized cell cultures, SHE cells were cultured as described below for continuous BrdU labeling. After transferring the cells into the regular growth medium, they were treated with DES (20 µ*M*) for 7 h at three different time intervals which corresponded to different cell cycle phases (table 1). Fixation was performed 30 h after transfer into culture medium. Fixation, staining and evaluation were as described above.

Continuous BrdU Labeling

SHE cells were synchronized by growth to confluency followed by a reduction in FCS to 0.5% for 24 h. After transferring the cells to regular growth medium (15% FCS) at lower cell density, the culture medium was then supplemented with 100 µ*M* BrdU (unless stated otherwise) and DES as indicated in the text. Since BrdU is a strong photo- and radiosensitizer, cell cultures were protected from light of short wavelengths by wrapping all culture flasks with aluminium foil, and by performing all further steps under dimmed light. After the appropriate period of culture (indicated in Results), cells were harvested by washing in calcium/-magnesium-free phosphate-buffered saline and treatment with trypsin solution, resuspended in culture medium supplemented with 10% DMSO and stored at –20°C in the dark.

Cell Staining and Flow Cytometry

Cells, which were stored at –20°C, were thawed. Then they were centrifuged and the cell pellet resuspended in staining buffer at a density of 4×10^5 to 10^6 cells/ml and cells were incubated for 15 min in the dark [5–7]. The staining buffer contained 154 m*M* NaCl, 100 m*M* Tris-HCl (pH 7.4), 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 0.1% NP_{40} and 1.2 µg/ml Hoechst 33258. Thereafter, ethidium bromide (final concentration 2 µg/ml) was added and, after a further incubation period of 15 min, samples were analyzed by flow cytometry. This technique was performed on an arc lamp-based epi-illumination system (Partec PAS II, Münster, Germany). Appropriate excitation light is selected with an UG 1 and a BG 38 filter (Schott) and an FT 450 dichroic mirror. Hoechst fluorescence is obtained with a K 45 filter and an FT 510 dichroic mirror, and ethidium bromide fluorescence is collected with a K65 LP filter. Bivariate cytograms were transferred to an MS-DOS-operated personal computer and analyzed using the Multi2D-package (Phoenix Flow Systems, San Diego, Calif., USA). Data analysis was according to the procedures described by Rabinovitch et al. [6].

Table 1. Micronucleus induction by DES (20 µ*M*) in synchronized SHE cell cultures

Treatment, time, h	Cell cycle phase	Micronuclei/ 2,000 cells
Untreated		29.6 ± 1.8
2.5–9.5	G0/G1	29.3 ± 2.5
10–17	S	34.6 ± 2.1
17.5–24.5	(S), G2, (M)	42.8 ± 2.8

Cells were treated for 7 h in different cell cycle phases. Treatment time is given as the time after transfer into normal growth medium. The corresponding cell cycle phases are also given.

Results and Discussion

With increasing doses of DES, micronucleus formation in SHE cells rises (fig. 1). Even beyond the dose at which the highest rate of micronucleus formation was observed, no fragmentation of nuclei occurred. At the highest dose applied the relative number of micronuclei was less than that found with the previous dose. This type of dose-response relationship suggests that the extent of cell proliferation, more specifically an inhibition in the rate of mitosis at higher substance doses, may play a role in the expression of micronucleus formation. Figure 2 shows the time course of micronucleus formation after exposing SHE cells to DES, which agrees with data published previously [3]. Maximal micronucleus formation was observed 12 h after removal of DES. The early formation of micronuclei after exposure suggests that a short time frame is needed between the induction of the micronucleus-forming event and its actual expression. The decline in micronucleus frequency at later time points can be interpreted as a dilution of micronucleus-containing cells by proliferating cells without micronuclei. To clarify fur-

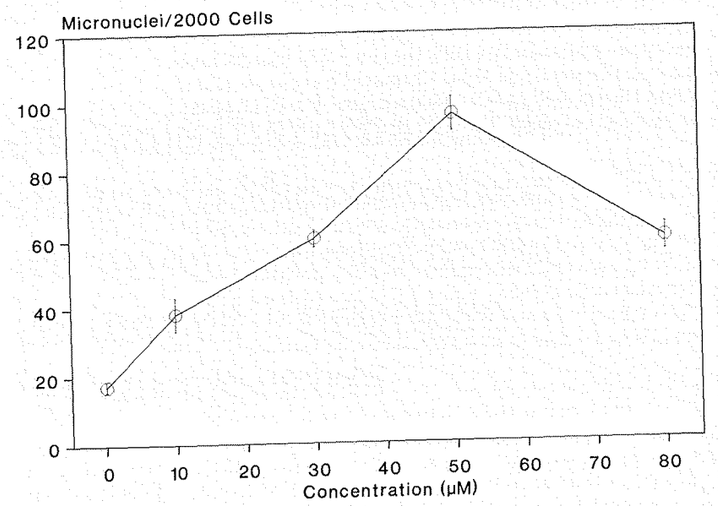

Fig. 1. Induction of micronucleus formation in SHE cells by DES. Cells were treated for 5 h and fixed 12 h later.

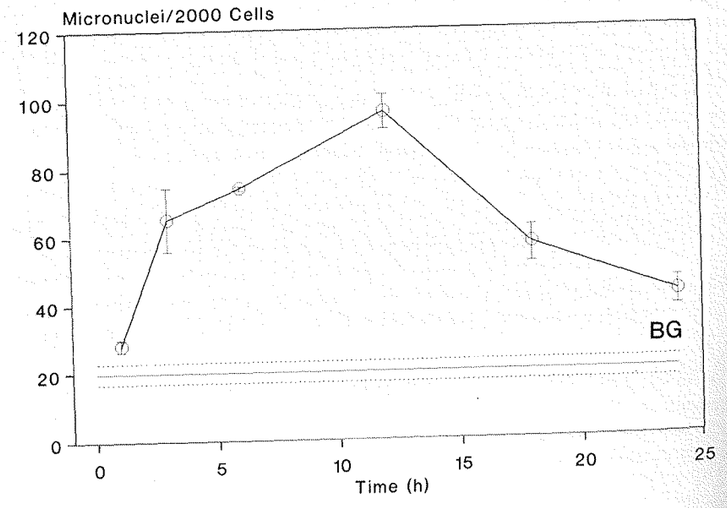

Fig. 2. Time course of micronucleus induction by DES (50 μ*M*, 5 h) in SHE cells. Zero time corresponds to the moment of DES removal. The area marked 'BG' (background) indicates the mean of the spontaneous micronucleus frequency.

ther the possible relationship between cell cycle progression and micronucleus formation, we analyzed the influence of DES on cell cycle traverse of SHE cells by continuous BrdU labeling and bivariate flow cytometry.

Figure 3 shows the bivariate cytograms obtained from a culture exposed to 12.5 μ*M* DES and from an untreated control after 30 h in the presence of BrdU. Each signal dot originally represents a single cell; at areas where many signals concur clusters emerge. The abscissa shows blue-green Hoechst fluorescence, whereas the ordinate depicts red ethidium bromide fluorescence. The amplification settings of the flow cytometer are adjusted such that the cluster of resting cells (G0/G1) appears at the right-hand lower corner of the cytogram. Upon entry into the S phase, cells increase their DNA content while incorporating BrdU, which quenches the Hoechst fluorescence. The intensity of

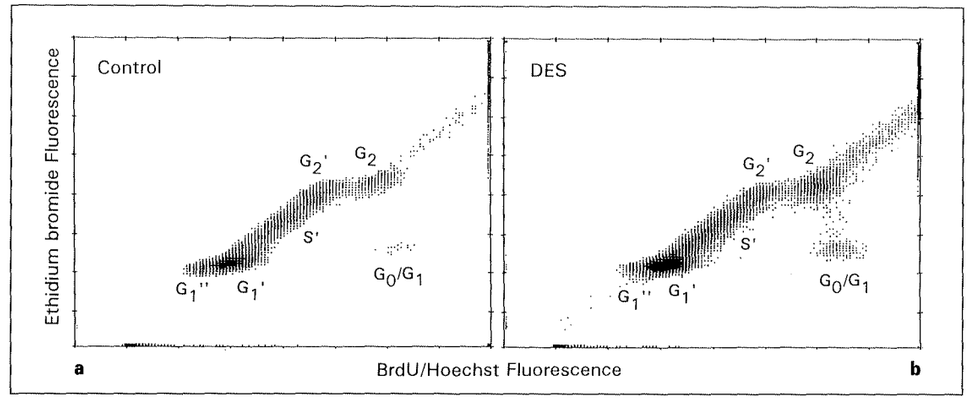

Fig. 3. Bivariate Hoechst/ethidium bromide cytograms of SHE cell cultures continuously labeled with BrdU. Cytograms obtained with a control (**a**) and DES-treated (12.5 μ*M*) cultures (**b**) are shown. The abscissa in each panel represents BrdU-quenched Hoechst fluorescence, while the ordinate shows unquenched ethidium bromide fluorescence. Further explanation is given in the text.

DNA-bound ehtidium bromide fluorescence is unaffected by BrdU incorporation. As a result, the fluorescence intensity of BrdU-incorporating S-phase cells decreases on the Hoechst axis and increases on the ethidium bromide axis. The cluster representing cells in the G2 compartment of the first cycle thus emerges left upwards from the G0/G1 cluster. Upon mitosis the fluorescence intensities of the resulting G1 cells are halved on both axes, which separates the G1 cells of the second cycle (G1′) from the resting cells (G0/G1). A trail of second-cycle S-phase cells emerges from the G1′ cluster. A phenomenon similar to that seen with the first round of BrdU incorporation develops during this S phase. Due to bifiliary substitution the second S-phase trail moves to the right as opposed to the leftwards direction of the first-cycle (unifiliary substituted) S-phase trail. Thus, the cluster of second-cycle G2 cells appears to the left of the first-cycle G2-phase cells. As a result the G1 cells after the second mitosis appear in a cluster left from the signals representing the G1 cells after the first mitosis. Thus, a cytogram develops which allows one to distinguish cells according to the G1, S and G2 compartment of the three successive cell cycles. The striking difference between the untreated control and the DES-exposed culture is the relative number of signals in the G2 cluster of the first cycle. Since more cells accumulate in the first-cycle G2 compartment in the DES-exposed culture, fewer cells reach the third cycle G1 phase (fig. 3).

By framing of the areas covered by the signals representing a cell cycle or a single cluster, the relative number of cells in this area can be quantified. This procedure allows one to assess the influence of a drug upon cell-cycle distribution. Together with the relative rate of growth of cell cultures exposed to various concentrations of the drug, a 'cell-cycle fingerprint' can be generated for each drug [8]. Figure 4 shows the cell-cycle fingerprint of DES. The distance between two successive curves gives the percentage of cells that accumulated in that cell-cycle compartment under exposure to the drug. A widening between two curves thus indicates an inhibition of cell transit through this particular cell-cycle compartment [8]. Exposure to increasing concentrations of DES induces an accumulation of cells in the G0/G1, S and G2 phase. Thus, DES does not exhibit cell-cycle compartment specificity, although the G2 phase clearly is the most sensitive cell-cycle stage. At a concentration of 25 μ*M* DES, cell growth was reduced to zero. This means that, under the continuous treatment used here, a total cell-cycle arrest was reached. Therefore, concentrations in the range of 12.5–20 μ*M* DES are most suitable for experiments with continuous DES exposure.

To elucidate the cell-cycle mechanism underlying DES action, time-resolved analysis of cell-cycle distributions was necessary. Plotting of the relative numbers of cells in each compartment versus the time of observation allows one to follow the kinetics of cell-cycle compartment traverse in the culture under observation [5, 6, 9]. Extrapolation of the curves representing entry into and exit from a given cell-cycle phase to the time point of zero cells in the compartment allows determination of the minimal duration of this cell-cycle stage [5, 6]. Extrapolation of two suc-

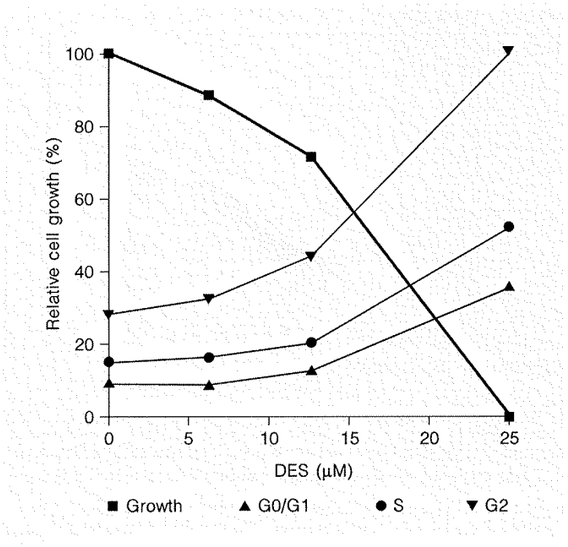

Fig. 4. Dose-response curves of cell cultures exposed to a series of DES concentrations for 30 h. For explanation see text. Cell labeling was with 100 μg/ml BrdU.

Table 2. Minimal duration of cell cycle compartments (h)

Compartment	Control	DES exposed
G0/G1	8.8±0.7	10.0±0.2
S	8.8±0.1	8.7±0.2
G2	2.3±1.1	2.5±0.1

Data are computed from exit kinetic curves as described in the text. The DES concentration was 20 μ*M*.

Table 3. Fraction of cells arrested in each cell cycle compartment (% of cells)

Compartment	Control	DES exposed
G0/G1	0.4±0.1	5.2±0.1
S	1.5±0.1	2.8±0.1
G2	2.0±0.2	15.9±0.2

Data are computed from exit kinetic curves as described in the text. The DES concentration was 20 μ*M*.

cessive curves to infinite time will reveal the relative number of cells which entered a given cell-cycle stage without exiting from it. In other words, the fraction of cells irreversibly arrested in each cell-cycle compartment can be deduced from this plot.

Applying these algorithms to our experiments generated the computed minimal duration of each cell-cycle compartment and the fraction of arrested cells for the untreated control and the cell cultures continuously exposed to 20 μ*M* DES (tables 2, 3). The drug treatment induces a delay in cell activation (increase in the G0/G1 duration from 8.8 to 10.0 h). In addition, exposure to DES impairs cell activation (the G0/G1 arrest fraction rises from 0.4 to 5.2%), S-phase transit and predominantly G2-compartment traverse (increase from 2.0 to 15.9%).

Disturbance in cell activation is a common result of exposure to cytotoxic agents [8]. In accordance with this general experience, DES provoked G0/G1 delay and arrest. G0/G1 arrested cells are not likely to contribute to micronucleus-forming events.

Impaired S-phase transit was unexpected since DES is not known to bind to DNA. However, DES as well as 5-azacytidine induced DNA hypomethylation in SHE cells [10]. In addition, 5-azacytidine has been shown to perturb G2 phase transit [11] and to induce micronuclei in SHE cells [12, 13]. Cytogenetic analysis revealed that the majority of micronuclei induced by 5-azacytidine treatment did not contain whole chromosomes, but chromatin fragments [12]. The micronuclei formed upon exposure to DES, on the other hand, mostly contained whole chromosomes [3]. Therefore, the possible relationship between DNA methylation and micronucleus formation remains unclear.

The most striking change in cell-cycle progression was an impaired traverse of the G2 phase. The short time period between the micronucleus-inducing event and expression of micronuclei seen in the time course of micronucleus induction (fig. 2) suggests that the micronucleus-forming cells were in the S or G2 phase of the cell cycle at the time of treatment with DES.

To study micronucleus induction, unsynchronized cultures were treated with DES for 5 h, whereas in flow cytometric experiments a synchronized cell population was needed [6]. This well-established method of continuous BrdU labeling required continuous DES treatment. Therefore, lower DES doses were applied. In a final experiment we performed a micronucleus experiment under conditions similar to those used for the flow cytometric experiments. SHE cell cultures were synchronized and a DES concentration of 20 μ*M* was used. However, continuous DES treatment could not be applied, since all cells would have formed micronuclei during the first mitosis after treatment and identification of the sensitive phases in which the micronucleus-inducing event occurred would not have been possible. Therefore, a treatment duration of 7 h was chosen. In addition to one untreated control culture, one culture was treated from 2.5 to 9.5 h (corresponding to the G0/G1 phase) after transfer into cell culture medium, one from 10 to 17 h (corresponding to the S phase) and one from 17.5 to 24.5 h. The last treatment time corresponded to S-, G2-, M- and G1′-phase cells (with G1′-phase cells being unable to form micronuclei within the observation period) and was the only treatment time in which significant amounts of G2-phase cells were present during DES exposure. The results of this experiment are shown in table 1. Although treatment of SHE cells caused a small increase in micronucleus frequency in the second treatment protocol, a much greater increase could be observed after treatment at 17.5–24.5 h (third treatment protocol, predominantly G2-phase cells). This supports the hypothesis that the G2 phase is a sensitive stage for micronucleus induction by DES. The small increase in micronucleus formation during the second treatment protocol can be explained in different ways. First, a minor fraction of cells may have traveled faster and entered the G2 compartment during exposure to DES. In this case all micronucleus-forming cells would have been in the G2 phase. Second, DES may not have been quantitatively removed from cells, which then may have carried the drug from the S into the G2 compartment. In this case, again, all cells experienced the micronucleus-inducing effects while residing in the G2 phase. Third, cells in the S phase may exhibit a micronucleus-forming property when exposed to DES. This property has to be restricted to either a late stage of the S compartment or would be rather weak. A final conclusion can as yet not be drawn from the data presented here.

It can be envisaged that DES may act by the formation of oxygen free radicals or a related highly reactive species. Oxygen free radicals have been shown to exert a synergism with BrdU, the agent used in this study to label proliferating cells [7]. To assess such a possible synergism we varied the BrdU concentration between 70 and 350 μ*M* in an untreated control and a 12.5-μ*M* DES-exposed culture. In both cases parallel growth-inhibition curves were obtained (results not shown). Therefore, a synergism between DES and BrdU can be excluded. Moreover, DES is not likely to exert its cell cycle kinetic and micronucleus-forming effects via the formation of DNA damage involving free radical attack.

The concurrence between strong cell cycle-disturbing effects caused by DES and the formation of micronuclei suggests that impairment of G2 transit may reflect a process ultimately leading to micronucleus formation. Identification of critical processes taking place in the G2 phase of the cell cycle, which upon perturbation lead to micronucleus formation, may present an avenue for future research.

References

1 IARC: Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol 21: Sex Hormones (II). Lyon, International Agency for Research on Cancer, 1979.
2 Tsutsui T, Maizumi H, McLachlan JA, Barrett C: Aneuploidy induction and cell transformation by diethylstilbestrol: a possible chromosomal mechanism in carcinogenesis. Cancer Res 1983;43:3814–3821.
3 Schiffmann D, DeBoni U: Dislocation of chromatin elements in prophase induced by diethylstilbestrol: A novel mechanism by which micronuclei can arise. Mutation Res 1991;246:113–122.
4 Schiffmann D, Reichert D, Henschler D: Induction of morphological transformation and unscheduled DNA synthesis in Syrian hamster embryo fibroblasts by hexachlorobutadiene and its putative metabolite pentachlorobutenoic acid. Cancer Lett 1984;23:297–305.
5 Rabinovitch PS: Regulation of human fibroblast growth rate by both noncycling cell fraction and transition probability is shown by growth in 5-bromodeoxyuridine followed by Hoechst 33258 flow cytometry. Proc Natl Acad Sci USA 1983;80:2951–2955.
6 Rabinovitch PS, Kubbies M, Chen YC, Schindler D, Hoehn H: BrdU-Hoechst flow cytometry: A unique tool for quantitative cell cycle analysis. Exp Cell Res 1988;174:309–318.
7 Poot M, Rabinovitch PS, Hoehn H: Bromodeoxyuridine amplifies free-radical-mediated DNA damage. Biochem J 1989;261:269–271.
8 Poot M, Schuster A, Hoehn H: Cytostatic synergism between bromodeoxyuridine, bleomycin, cisplatin and chlorambucil demonstrated by a sensitive cell kinetic assay. Biochem Pharmacol 1991;41:1903–1909.
9 Smith JA, Martin L: Do cells cycle? Proc Natl Acad Sci USA 1973;70:1263–1267.
10 Pechan R, Schiffmann D: Diethylstilbestrol induces DNA-hypomethylation in Syrian hamster embryo fibroblasts throughout the course of neoplastic transformation. Naunyn Schmiedebergs Arch Pharmacol 1987;335 (suppl):R30.
11 Poot M, Koehler J, Rabinovitch PS, Hoehn H, Priest JH: Cell kinetic disturbances induced by treatment of human diploid fibroblasts with 5-azacytidine indicate a major role for DNA methylation in the regulation of the chromosome cycle. Hum Genet 1990;84:258–262.
12 Stopper H, Pechan R, Schiffmann D: 5-Azacytidine induces micronuclei in and morphological transformation of Syrian hamster embryo fibroblasts in the absence of unscheduled DNA synthesis. Mutation Res 1992;283:21–28.
13 Stopper H, Körber C, Schiffmann D, Caspary WJ: Cell cycle dependence of 5-Azacytidine induced micronuclei and mitotic disturbances in mammalian cells. Mutation Res 1993;300:165–177.