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Endoreduplication induced in cultured Chinese hamster cells by different anti-topoisomerase II chemicals Evidence for the essential contribution of the enzyme to chromosome segregation

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Abstract

With the ultimate purpose of testing the hypothesis that, as shown in yeast mutants, any malfunction of DNA topoisomerase II might result in aberrant mitosis due to defective chromosome segregation, we have chosen three chemicals of different nature, recently reported to catalytically inhibit the enzyme. The endpoint selected to assess any negative effect on the ability of topoisomerase II to properly carry out decatenation of fully replicated chromosomes in the G2/M phase of the cell cycle was the presence of metaphases showing diplochromosomes as a result of endoreduplication, i.e. two successive rounds of DNA replication without intervening mitosis. The anti-topoisomerase drugs selected were the anthracycline antibiotic and antineoplastic agent aclarubicin, the respiratory venom sodium azide, and 9-aminoacridine, a chemical compound with planar topology capable of intercalation between DNA bases. Our results show that the three chemicals tested are able to induce endoreduplication to different degrees. These observations seem to lend support to the proposal that topoisomerase II plays a central role in chromosome segregation in mammalian cells.

Keywords: DNA topo II; Endoreduplication; Aclarubicin; Sodium azide; 9-Aminoacridine

1. Introduction

The phenomenon of endoreduplication, which is rather common in plants [1], but only rarely observed to occur spontaneously in animals, has drawn a lot of attention from both cytogeneticists and investigators

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of cell-cycle genetics and biochemistry. In spite of this, no clear or unique mechanism of induction of endoreduplication has been proposed, which is partly due to the variety of agents able to induce it, but also to the various cell types in which it has been described so far. Diplochromosomes, which are the visible mitotic manifestation of this striking phenomenon, are made up of four chromatids held together, instead of the normal two, as a result of the occurrence of two successive rounds of DNA replication without intervening mitosis, i.e. segregation of daughter chromatids [2,3].

Focusing on cell-cycle stages susceptible to induction of endoreduplication by chemicals, the G2-mitosis phase appears to be the most sensitive [4–6]. Recently, in human fibrosarcoma cells, it has been reported that $p21^{waf1/Cip1/Sdi1}$-induced growth arrest is associated with depletion of mitosis-control proteins, leading to abnormal mitosis and endoreduplication in recovering cells [7]. This observation seems to be consistent with the role of the cyclin-dependent kinase (CDK) inhibitor p21 as an integral part of cell growth arrest associated with DNA damage, which in turn often involves the triggering of endoreduplication. One of the essential mammalian proteins whose expression might be inhibited by p21 is likely to be topoisomerase II (topo II), which plays a central role in chromosome segregation [8]. In mammalian cells, the existence of a temporary G2 topo II-dependent checkpoint that regulates entry into mitosis has been proposed [9].

Yeast temperature-sensitive mutants of DNA topo II fail to carry out both chromosome condensation and anaphase chromatid segregation [10–12]. In mammalian cells, on the other hand, even though the lack of available mutants represents a drawback to test the importance of the enzyme for chromosome segregation, it has been shown that topo II poisoning [13] or catalytic inhibition [14] prevents chromosome segregation and can result in endoreduplication [3].

Supporting the importance of topo II for chromosome segregation at mitosis, we have recently reported on a high yield of endoreduplication induced by the bisdioxopiperazine ICRF-193, a topo II catalytic inhibitor [15]. On the other hand, we have also recently found that endoreduplication is readily induced in AA8 Chinese hamster cells treated for two consecutive cell cycles with different halogenated nucleosides, namely 5-chlorodeoxyuridine (CldU), 5-iododeoxyuridine (IdU), and 5-bromodeoxyuridine (BrdU). Interestingly however, treatment for just one cell cycle did not lead to a similar increase in endoreduplication [16], most likely pointing to a major relative importance of template DNA as compared to the nascent molecule for proper chromosome segregation.

As regards to the specificity of the endoreduplication assay, taking into account the data mentioned above, we have recently elaborated a comprehensive model for induced endoreduplication [17], which supports the idea of the importance of inhibition of topo II function. In the present investigation, we have tested the ability of three drugs, reported to be capable of acting against topo II through different mechanisms, to induce endoreduplication: the topo II catalytic inhibitor aclarubicin [18], the cellular ATPase poison sodium azide, also considered as a catalytic inhibitor of the enzyme [19,20], and the planar DNA-intercalative drug 9-aminoacridine [21]. The results show that all three drugs are cytotoxic, show a clear inhibitory effect on topo II catalytic activity, and are able to induce endoreduplication to different degrees. These positive results, which seem to support the importance of fully operative topo II for chromosome segregation, are discussed.

2. Materials and methods

2.1. Culture conditions

The parental cell line AA8 (purchased from the American Type Culture Collection (ATCC), USA) was grown in monolayer in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2×10^{-3} M L-glutamine and the antibiotics penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were grown in the dark at 37 °C in a 5% CO_2 atmosphere. On regular testing, cell cultures were found to be free from mycoplasma.

2.2. Drugs

Both aclarubicin and 9-aminoacridine were obtained from Sigma (USA), while sodium azide was purchased from Merck (Germany). Aclarubicin was prepared in ethanol and the other drugs were dissolved in distilled water and directly added to the culture

medium. Concentrations and treatment times of the three chemicals were comparable to those reported to inhibit topo II catalytic activity in mammalian cells.

2.3. Growth-inhibition assay

Cells in exponential growth phase were harvested using trypsin-EDTA (Gibco BRL) and resuspended in complete culture medium. They were seeded at 5×10^3 cells/100 μl in 96-well microtitre plates (Nunc). After 24 h, to allow attachment, the cells were incubated for 48 h in the presence of different concentrations of the three compounds diluted in tissue culture medium (100 μl, final volume). The dose range tested was from 0.006 to 0.49 μM for aclarubicin, from 0.25 to 50 mM for sodium azide and from 2 to 70 μM for 9-aminoacridine.

For growth inhibition studies, the sulforhodamine B (SRB) assay was used as described previously [22,23]. Briefly, 50 μl/well cold 50% trichloroacetic acid (TCA) (final concentration 10%) was added to the culture and incubated at 4 °C for 1 h, to precipitate the proteins and fix the cells. The supernatant was then discarded and the plates were washed five times with deionized water and air-dried. The cells were stained with 0.4% SRB (100 μl/well) dissolved in 1% acetic acid for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid and then the plates were air-dried. The stained protein was solubilised in 100 μl/well of 10 mM unbuffered Tris base by shaking. The optical density was read at 492 nm using a microtitre plate reader (ELISA). Each experiment was performed in triplicate.

2.4. Preparation of nuclear extracts

Topo II activity in nuclear extracts from AA8 cells was obtained as described by Heartlein et al. [24]. Approx. 10^7 cells were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris–HCl, pH 7.5, 0.05 M $MgCl_2$ and 1% Triton X-100 and thoroughly vortexed to lyse the cells. Nuclear pellets were obtained by centrifugation at $1800 \times g$ (Eppendorf centrifuge) for 5 min at 4 °C. Nuclei were then washed in 1 ml of nucleus wash buffer (5 mM potassium phosphate buffer, pH 7.5, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM β-mercaptoethanol and 0.5 mM dithiothreitol (DTT)). The nuclei were then pelleted as described above and resuspended in 50 μl of nucleus wash buffer, and 50 μl of 4 mM EDTA was added. Following incubation at 0 °C for 15 min, the nuclei were lysed by adding 100 μl of 2 M NaCl, 20 mM Tris–HCl, pH 7.5, 10 mM β-mercaptoethanol and 1 mM PMSF. Following a 15-min incubation at 0 °C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, 50 mM Tris–HCl, pH 7.5, 10 mM β-mercaptoethanol and 1 mM PMSF was added. The suspension was incubated for a further 40-min period at 0 °C. The supernatant from a 30-min centrifugation at $11{,}200 \times g$ at 4 °C was then collected. The total protein concentration in each extract [25] was determined in a Beckman DU-64 spectrophotometer by the Bio-Rad protein assay (Bio-Rad Laboratories). The extracts were kept at −80 °C for no longer than a month.

2.5. Topoisomerase II activity in nuclear extracts

Topo II activity in nuclear extracts was assayed using TopoGen (Columbus, OH, USA) assay kits based upon decatenation of kinetoplast DNA (kDNA). Reaction mix was incubated with different doses of the tested agents. After a 40-min incubation at 37 °C, the samples were loaded onto 1% agarose gels and subjected to electrophoresis for 2.5 h at 100 V. Finally, gels were stained with 0.5 μg/ml ethidium bromide, destained (30 min) in distilled water and photographed using a standard photodyne set.

2.6. Induction of endoreduplication

Actively growing cultures AA8 cells were treated with aclarubicin (3 h), 9-aminoacridine (2h) or sodium azide (1h). The range of concentrations used were 0.006–0.25 μM for aclarubicin, 0.25–10 mM for sodium azide and 2–50 μM for 9-aminoacridine. After treatment the cultures were thoroughly washed and maintained in fresh medium for 18 h to allow them to recover. In every experiment, cultures that did not receive any treatment served as controls. Colcemid (2×10^{-7} M) was finally added for the last 3 h of recovery to all the cultures for metaphase arrest.

The flasks were shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 0.075 M KCl for 2 min (hypotonic treatment), fixed in methanol:acetic acid (3:1) and dropped onto clean

glass microscope slides. The slides were stained with 3% Giemsa in phosphate buffer pH 6.8 and mounted in DPX. Two thousand metaphases per experimental point were scored and classified as normal or as having diplochromosomes. All experiments were carried out in triplicate.

3. Results

The compounds aclarubicin, sodium azide and 9-aminoacridine were selected for this study due to their reported action as topo II catalytic inhibitors in a wide sense, even though they have been proposed

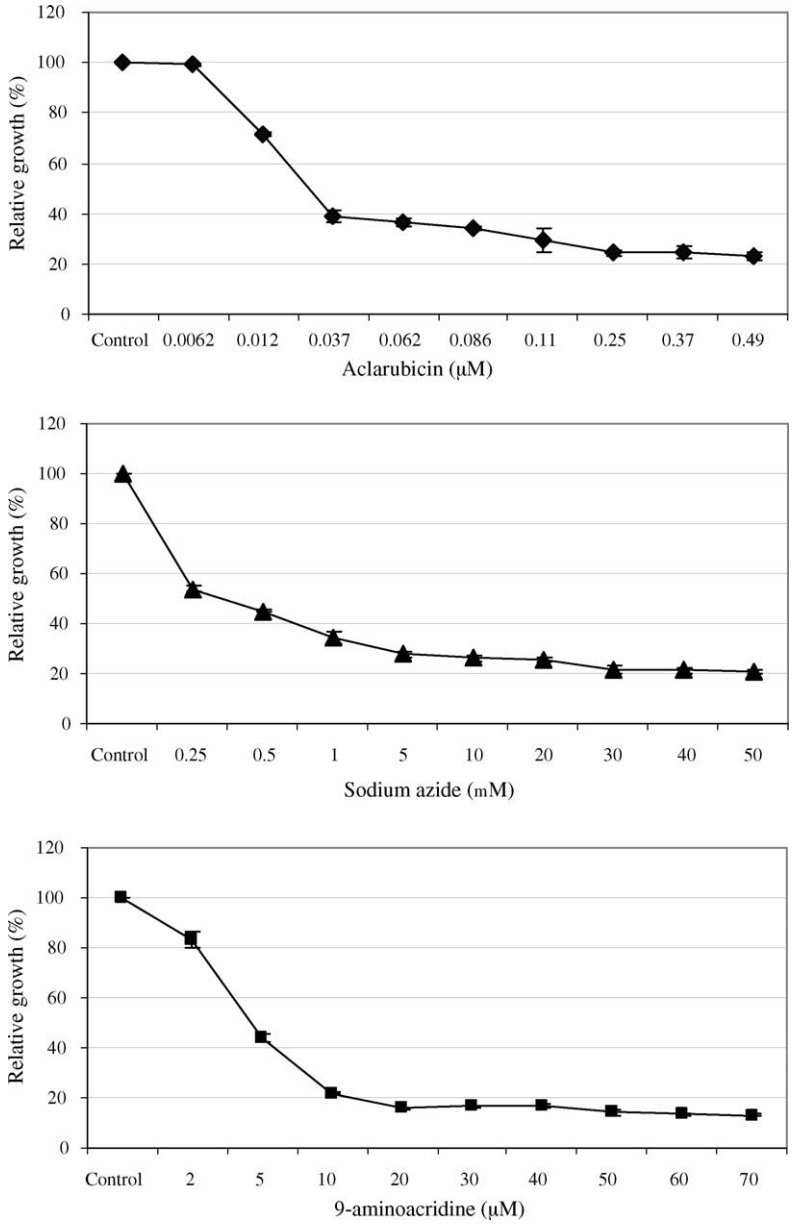

Fig. 1. Effects of different concentrations of aclarubicin, sodium azide and 9-aminoacridine on cell viability in AA8 Chinese hamster ovary cells, as shown by the SRB assay. Bars indicate standard deviations of results from three independent experiments.

to interfere with the nuclear enzyme by different mechanisms. Assessment of the cytotoxic activity of these drugs as well as their ability to efficiently inhibit the catalytic activity of topo II was carried out before analyzing the possible induction of endoreduplication in AA8 Chinese hamster ovary fibroblasts.

In all cases, the highest concentration evaluated for each compound in the endoreduplication study was that which was not overtly toxic or clastogenic for the cycling cells. This is especially important for the respiratory venom sodium azide, which shows a high toxicity.

3.1. Induced cytotoxicity

The anthracycline aclarubicin is an intercalative antibiotic and antineoplasic agent that efficiently binds to DNA, leading to a secondary inhibition of topo II catalytic activity on DNA. We have recently reported on the different efficiencies of aclarubicin to induce cytotoxicity and chromosome and DNA damage in both V79 and radiosensitive *irs2* Chinese hamster lung fibroblasts [26]. On the other hand, given its planar topology, the intercalative nature of 9-aminoacridine is also well known [21,28]. Concerning sodium azide, it has been reported that it is a respiratory venom that interferes with both ATP levels and ATP cellular catabolism.

Fig. 1 shows the results of the cell viability test using the SRB assay: all compounds tested were able to induce very efficiently an inhibitory effect, with different profiles depending on the compound tested. Aclarubicine was most efficient in inhibiting cell growth, being able to exert its cytotoxic effect at extremely low doses, while 9-aminoacridine had an intermediate cytotoxic effect. The respiratory venom sodium azide was the least effective (Fig. 1).

3.2. Inhibition of topo II catalytic activity

The enzyme capacity to decatenate double-stranded catenated kinetoplast DNA (kDNA) was the endpoint used to monitor inhibition of topo II catalytic activity by the two intercalative compounds aclarubicin and 9-aminoacridine as well as by sodium azide. As can be seen in Fig. 2, in the absence of any drug treatment, topo II activity recovered in nuclear extracts from AA8 cells was able to efficiently decatenate the catenated DNA substrate as shown by the release of closed minicircles. When inhibition of this activity by increasing

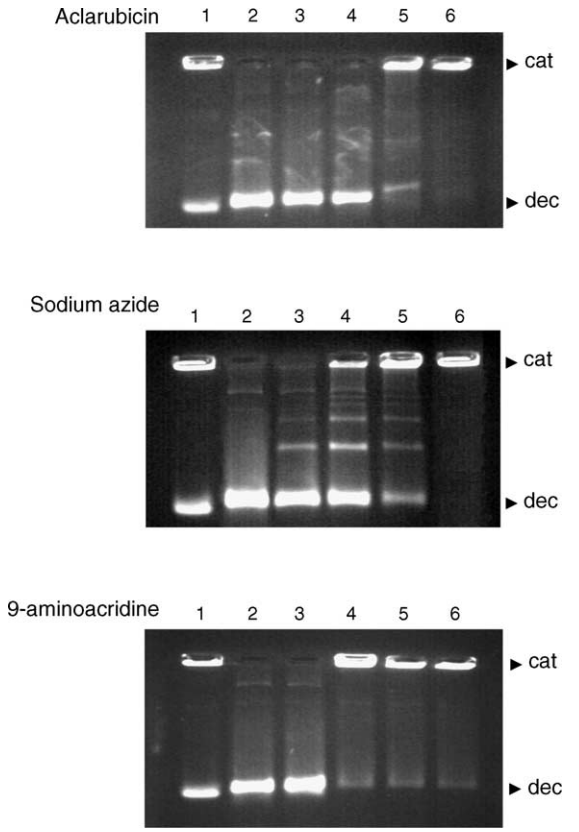

Fig. 2. Effectiveness of different doses of aclarubicin, sodium azide and 9-aminoacridine to inhibit the topo II catalytic activity. Nuclear extracts were obtained as described in Section 2 and their ability to decatenate catenated kinetoplast DNA was assayed by gel electrophoresis. Lane 1: marker catenated (cat) and decatenated (dec) DNA; lane 2: control, not treated with any compound; lanes 3–6: treated with increasing concentrations of aclarubicin (1.2, 2.4, 6.1 and 12.3 μM, respectively), sodium azide (10, 20, 40 and 60 mM) and 9-aminoacridine (20, 40, 60 and 80 μM).

concentrations of the three compounds was tested, a dose-dependent inhibitory effect was observed, as shown by a progressive increase in the amount of catenated DNA substrate remaining in the wells (Fig. 2). On the basis of these results, different dose intervals were selected for the experiments aimed to assess effects of the compounds on chromosome segregation.

3.3. Effects on chromosome segregation

In good agreement with the results presented above (see Fig. 2), the drug 9-aminoacridine was recently

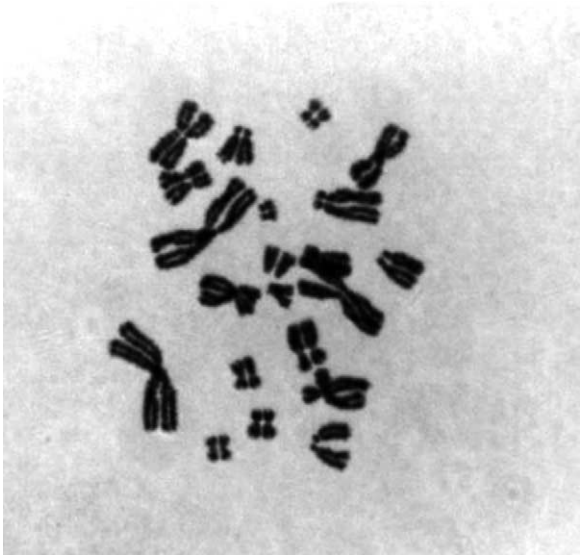

Fig. 3. AA8 Chinese hamster endoreduplicated cell at metaphase after treatment with the topo II catalytic inhibitor aclarubicin, showing the characteristic diplochromosomes.

recognized as a topo II catalytic inhibitor, based upon different criteria such as its antagonism with the topo II poison etoposide [21]. Sodium azide, on the other hand, was also shown to exert a catalytic inhibition of DNA topo IIα [19,20], most likely due to the high dependence of the enzyme on its ATPase function [27].

We have evaluated the possible induction of endoreduplication as a result of treatment with the three test compounds. As can be seen in Figs. 3 and 4, all three chemicals were able to induce endoreduplication to different degrees, with 9-aminoacridine being the most efficient, with an optimum activity at 30 μM yielding roughly 25% mitoses showing diplochromosomes, sodium azide being the least active, and aclarubicin showing an intermediate effect (Fig. 4). On the other hand, whereas for aclarubicin and 9-aminoacridine a dose-dependent effect was observed for a range of doses up to the cytotoxic level, the effect of sodium azide appeared to be independent of the dose tested.

4. Discussion

As replication progresses, the double-stranded DNA molecules of sister chromatids inevitably become concatenated. This entanglement of DNA must be resolved in such a way that separation of the chromatids occurs as a pre-requisite for a proper anaphase segregation [29]. Due to its unique molecular mechanism of action that consists of breaking and resealing double-stranded DNA in a concerted fashion, the only known eukaryotic enzyme capable of decatenation is DNA topoisomerase II [27,29,30]. In fact, temperature-sensitive *Top 2* yeast mutants have shown the essential role of this enzyme in decatenating sister chromatids before anaphase begins [10–12]. Nevertheless, while topo II is required for chromosome condensation and chromatid segregation, it is clearly not needed for progression through the later stages of mitosis, such as cytokinesis [11,14].

Endoreduplication in eukaryotes is a process that involves DNA amplification without corresponding cell division [2,3,31]. While the molecular mechanisms are poorly understood, it is an endpoint of choice to assess any possible failure in the proper decatenation of replicated chromosomes before mitosis [3,13,14].

The ultimate purpose of the investigation reported here was to assess the ability of drugs of diverse chemical nature, considered to interfere with the catalytic activity of topo II through quite unrelated mechanisms [19,21,32], to induce endoreduplication in cultured Chinese hamster ovary cells.

The present study confirms that, regardless of the specific molecular mechanism, as reported previously by us [15] and others [3], poisoning or catalytic inhibition of topo II results in endoreduplication to different degrees. Topoisomerase ‘poisons’ are chemicals that cause DNA strand breaks through stabilization of topo II covalently bound to DNA in the intermediate form, the so-called *cleavable complex* [8], while chemicals classified as true catalytic inhibitors [32] are in general considered as non DNA-damaging. While direct evidence is still lacking, a likely explanation is that prevention of decatenation of replicated chromosomes by topo II leads to the subsequent failure to complete anaphasic segregation. Accordingly, any agent able to interfere with the enzyme function at any step of its catalytic cycle would negatively affect the proper anaphase segregation at mitosis. One of two possible different pathways is then to be taken by the cells: either commitment to follow a genetic program and proceed to apoptotic cell death [33,34], or to skip anaphase, initiate a new cell cycle (endocycle), and become polyploid or endoreduplicated cells. The latter need to undergo a further round of DNA replication to show up at the next

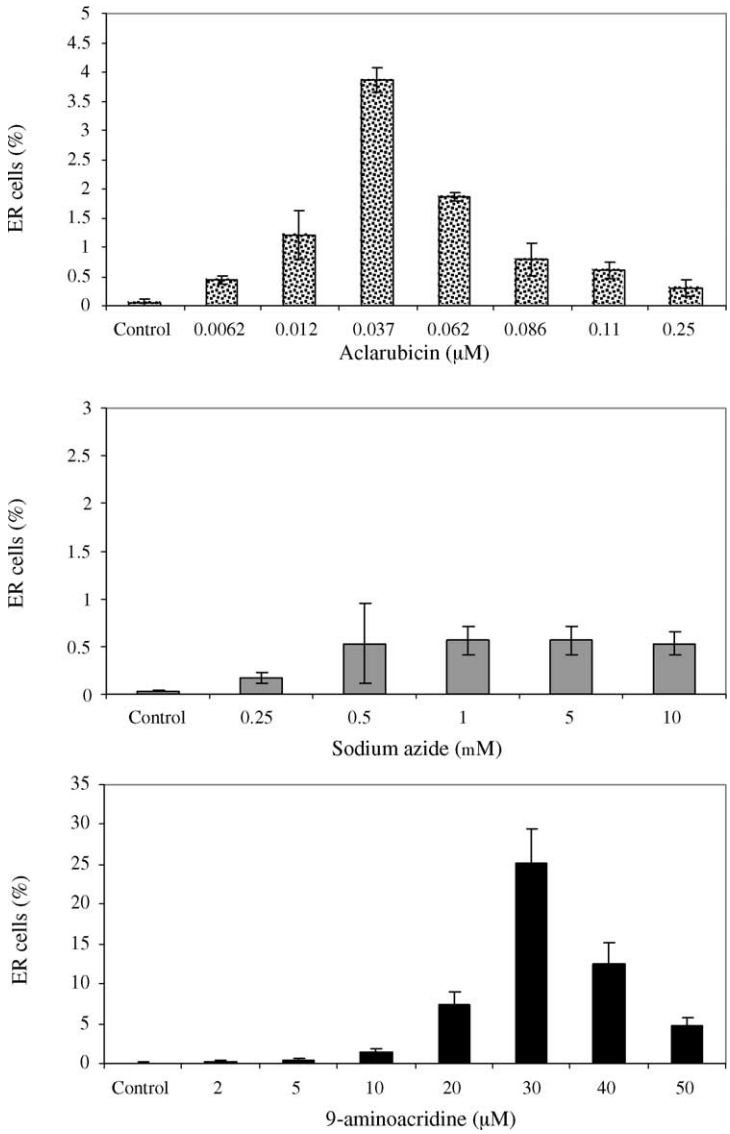

Fig. 4. Dose-dependent endoreduplication profile induced by the three chemicals selected on the basis of their catalytic inhibitory effect on DNA topo II. ER cells (%): the percentage of endoreduplicated mitoses among metaphases. Bars represent standard deviations (S.D.) of the mean of results from three independent experiments.

mitosis as metaphases made up of diplochromosomes [8,27].

The anthracycline aclarubicin is thought to inhibit topo II by blocking its binding to DNA [35]. While aclarubicin cannot stabilize the 'cleavable complex' like topo II poisons do, it eventually leads – through a molecular mechanism as yet poorly understood – to DNA and chromosome breakage [26,36] and cell death [26,35]. We have found that this planar intercalating molecule has also a clear dose-dependent effect on chromosome segregation, as is evident from the significant increase observed in the yield of endoreduplicated cells.

The respiratory venom sodium azide has been reported to render DNA topo II catalytically inactive, in such a way that the enzyme cannot initiate DNA

strand cleavage: the DNA/topoisomerase complex becomes insensitive to quinolones and other topo II inhibitors [19,20]. As sodium azide is well known to deplete cells of ATP through interference with mitochondrial electron transport [38] and to be inhibitory of some ATPases [37,38], the ATP-dependent function of topo II must be sensitive to the drug, while a high cytotoxicity as a whole can also be expected. We have observed a dose-dependent cytotoxic effect as a result of treatment with sodium azide. In spite of this, a moderate but consistent induction of endoreduplication was observed, as some cells succeeded to overcome the toxic effect and were able to undergo an endocycle and reach mitosis showing diplochromosomes.

Compared with aclarubicin, 9-aminoacridine is a smaller, but also planar molecule capable of intercalation between DNA bases [39]. It is well known that the ternary interactions between intercalating agent, DNA and topo II are complex. This complexity becomes evident from the observation that intercalating agents can either antagonize or actually enhance the formation of DNA double-strand breaks induced by topo II poisons [40,41]. In particular, 9-aminoacridine has been shown to behave as a topo II catalytic inhibitor that antagonizes the clastogenicity of the topo II poisons etoposide and doxorubicin [21]. While the exact molecular mechanism by which 9-aminoacridine exerts its catalytic inhibition has not been fully elucidated yet, we have again observed that, regardless of the specific step of the topo II catalytic cycle that results disturbed, any chemical capable of hindering the function of the enzyme appears to have a negative effect on chromosome segregation.

Taken as a whole, our results seem to support the idea that, in spite of the widely different levels of endoreduplication observed depending upon the anti-topo II chemical tested, a fully operative topo II is necessary for chromosome segregation before mitosis in mammalian cells, as shown earlier in yeast [10].

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