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High yield of endoreduplication induced by ICRF-193: a topoisomerase II catalytic inhibitor

Nuria Pastor, Maria José Flores, Inmaculada Domínguez, Santiago Mateos, Felipe Cortés*

Department of Cell Biology, Faculty of Biology, University of Seville, Avda Reina Mercedes 6, 41012 Seville, Spain

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Abstract

An uncommonly high yield of spontaneous endoreduplication is a feature of the CHO mutant EM9, besides its defective repair of single, as well as double-DNA strand-breaks and its extraordinarily elevated yield of sister chromatid exchanges (SCEs) after bromodeoxyuridine (BrdU) incorporation into DNA. Since the nuclear enzyme topoisomerase II (topo II) has been reported to be responsible for the segregation of daughter chromosomes during mitosis, in the present investigation we have made use of the bisdioxopiperazine ICRF-193, a topo II catalytic inhibitor that interferes with the normal turnover of the enzyme. In order to see whether both EM9 cells and its parental cell line AA8, which show differences in the spontaneous frequency of endoreduplicated cells are or not equally sensitive to the topo II catalytic inhibitor, both cell lines have been treated with a range of doses of the bisdioxopiperazine. Our results show that both cell lines respond to the treatment entering in an endoreduplication cycle, but the EM9 cells are extremely sensitive to the inhibition of topo II.

Keywords: Endoreduplication; Diplochromosomes; Topoisomerase II; ICRF-193; EM9

1. Introduction

DNA topoisomerases (topos) are conserved nuclear enzymes that catalyze a variety of topological changes of DNA during many cellular processes such as replication, transcription and recombination through transient cleavage of the molecule, strand passing and religation (for a review, see [1]). While topoisomerase I (topo I) breaks and rejoins one DNA strand at a time, topo II is able to do so with the two strands that make up duplex DNA. Both type I and II enzymes are proficient in relaxing supercoiled DNA in order to relieve torsional tension generated during replication and transcription, while only topo II can decatenate intertwined DNA molecules. This unique decatenating, as well as unknotting activity of DNA topo II is essential for segregating replicated daughter chromosomes. Apart from its important functional roles in chromosome condensation and segregation, topo II is a basic structural protein highly present in the nuclear matrix and chromosome scaffold [2].

Diplochromosomes, made up of four chromatids held together, instead of the normal two, are the visible mitotic manifestation of the rare, although sometimes spontaneous phenomenon of endoreduplication. This consists on two successive rounds of DNA replication without intervening mitosis, i.e. segregation of daughter chromatids [3,4].

* Corresponding author. Tel.: +34-95-4557039; fax: +34-95-4615780.
E-mail address: cortes@us.es (F. Cortés).

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A variety of agents either by disturbing cytoskeleton assembly, such as the spindle poisons colcemid, colchicin or concanavalin A [5,6] or by damaging DNA [7–10] have been reported to induce endoreduplication to different degrees. More recently, agents that interfere with topo II have been used to provide further evidence that the enzyme is required for separation of daughter chromosomes. As a result of these studies, it has been shown that both topoisomerase "poisons", i.e. chemicals that cause DNA strand-breaks through stabilization of topo II covalently bound to DNA in the intermediate form, the so-called cleavable-complex [11], as well as true catalytic inhibitors [12] are able to induce endoreduplication [4,13] due to prevention of decatenation of replicated chromosomes by topo II with the subsequent failure to complete a normal mitosis.

The Chinese hamster cell mutant EM9, which was isolated from its parental line AA8 on the basis of its sensitivity to the chemical mutagen ethyl methanesulfonate (EMS) and which is also radiosensitive, is well characterized by an extraordinarily high frequency of sister chromatid exchanges (SCEs) after bromodeoxyuridine (BrdU) substitution into DNA [14]. An interesting additional feature of this cell line that, in our opinion, deserves special attention is its relatively elevated yield of metaphases showing diplochromosomes that are observed after colcemid treatment to induce metaphase arrest [15]. While spindle poisons can induce endoreduplication, as mentioned above [5], given the short treatment time (about 2 h) in colcemid, it is concluded that this observation cannot be a consequence of the spindle poisoning, but must be an intrinsic feature of the EM9 cell line.

The purpose of this investigation was to carry out a comparative analysis of the efficiency of the topo II catalytic inhibitor ICRF-193, a bisdioxopiperazine, in the induction of endoreduplication in the EM9 cell line, as well as in the parental line AA8.

The result was an extraordinarily high frequency of metaphases showing diplochromosomes after ICRF-193 treatment in EM9 at doses that inhibit topo II, which was about 10 times that induced in parallel in the parental cell line AA8. This observation is discussed in terms of the possible role played by topo II in chromosome segregation, as well as in regard of the as yet poorly understood mechanism(s) leading to endoreduplication.

2. Materials and methods

2.1. Cell culture

The parental Chinese hamster cell line AA8 and the mutant EM9 were purchased from American type culture collection (ATCC), USA.

Both cell lines were grown as monolayers in McCoy's 5A medium (Bio-Whittaker) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and the antibiotics penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were cultured in a dark environment at 37 °C in an atmosphere containing 5% CO_2. On regular testing, cell cultures were found to be free from mycoplasma.

2.2. Preparation of nuclear extracts

Exponentially growing AA8 and EM9 cells were incubated for 3 h in the presence of different doses (1–10 μM) of the topo II inhibitor ICRF-193 (Biomol, Germany). After the treatment, the cells were processed to obtain extracts of nuclear proteins, while untreated control cells were also sampled in parallel for comparison. The procedure was basically that described by Heartlein et al. [16]. Approximately 1×10^7 cells were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris–HCl pH = 7.5, 0.05 M $MgCl_2$, 1% Triton X-100 and thoroughly vortexed to lyse the cells. Nuclear pellets were obtained by centrifugation at 2000 rpm (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 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, 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 were added. The suspension was incubated for a further 40 min at 0 °C. The supernatant from a 30 min centrifugation at 12,500 rpm at 4 °C was then collected. Total protein concentration in each extract was

determined in a Beckman DU-64 spectrophotometer by the Bradford protein assay [17] and extracts were kept at −80 °C for no longer than 1 month.

2.3. Topoisomerase II activity in nuclear extracts

Topo II activity in nuclear extracts was assayed using a TopoGen (Columbus, OH, USA) assay kit based upon decatenation of kinetoplast DNA (kDNA). The amount of nuclear extract protein from the different cell lines used in each assay was 100 ng. Reaction products were resolved using agarose gel electrophoresis of DNA. After incubation (40 min at 37 °C for topo II) the samples were loaded onto 1% agarose gels and subjected to electrophoresis for 2.5 h at 100 V (topo II assay). Finally, gels were stained with 0.5 μg/ml ethidium bromide, destained (30 min) in distilled water and photographed.

2.4. Induction of endoreduplication

Actively growing cultures of AA8 and EM9 cell lines were cultured for 3 h in the presence of ICRF-193 at concentrations ranging from 0.025 to 2 μM. After treatment the cultures were washed and maintained in fresh medium for 18 h to allow them to recover. For both cell lines, cultures that did not receive any treatment served as controls. Colcemid (2×10^{-7} M) was finally added for 3 h 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. A 2000 metaphases per culture were counted and classified as normal or as having diplochromosomes. All the experiments were carried out in triplicate.

2.5. Western blots

AA8 and EM9 control cells were harvested and lysed in a buffer consisting of 5 mM Tris–HCl, 50 mM EDTA, 0.5% Triton X-100, pH = 8 in the presence of protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin and 5 mg/ml leupeptin, all from Sigma). The protein content for each sample was quantified (see Section 2.2) and 30 μg from each sample were loaded on 7.5% SDS-polyacrylamide gels. After separation, the proteins were transferred onto immobilon polyvinylidene difluoride (PVDF) strips and blocked with 5% non-fat milk for 1 h at room temperature. PVDF strips were incubated with primary antibody anti-topo IIα (TopoGen, Columbus, USA) for 1 h at 37 °C. Finally, the peroxidase-conjugated anti-rabbit IgG (from Amersham) antibody was incubated with

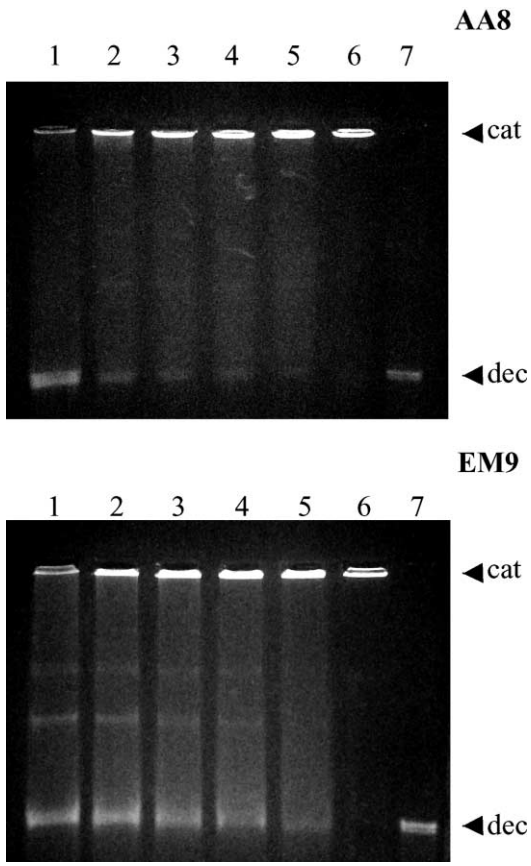

Fig. 1. Effectiveness of different doses of ICRF-193, ranging from 1 to 10 μM to inhibit the topo II catalytic activity. After treating the AA8 parental, as well as the CHO mutant EM9 cells in culture with the inhibitor, nuclear extracts were obtained and their ability to decatenate catenated kinetoplast DNA assessed by DNA gel electrophoresis. Lane 1: control non-treated with ICRF-193; lanes 2–5: treated with increasing concentrations of ICRF-193 (1, 2, 5, and 10 μM, respectively); lane 6: marker catenated (cat) kinetoplast DNA; lane 7: decatenated (dec) DNA marker.

the blot for 1 h at 37 °C and detected by the enhanced chemiluminescence method (Amersham). Densitometry was finally used for quantification of the Western blots (PCBAS 2.08).

3. Results

3.1. Inhibition of topoisomerase II catalytic activity in both AA8 and EM9 cell lines

The efficiency of topo II inhibition in both the CHO mutant EM9 and the parental line AA8 can be estimated from the loss of the ability of nuclear extracts to decatenate catenated kDNA as a consequence of cell treatment [15].

Fig. 1 shows the effectiveness of a 3 h treatment with the topo II catalytic inhibitor ICRF-193, a bisdioxopiperazine, in doses ranging from 1 to 10 μM, to modify the topo II activity as shown in nuclear extracts from cultured AA8 and EM9 cells.

As can be seen, both cell lines appear as sensitive to topo II catalytic inhibition by ICRF-193. While for both the parental AA8 and mutant EM9 cells, a concentration of 1 μM ICRF-193 appears as able to inhibit efficiently the topo II activity, a complete inhibition of the catalytic activity of the nuclear enzyme seems to be achieved for doses of 5 μM ICRF-193 and higher (Fig. 1).

Taking into account that we are dealing with total protein present in our nuclear extracts, Western blots were also analyzed in order to see whether both cell lines show any difference in the expression of topo II. Supporting our result on the similar sensitivity of both cell lines to the topo IIα catalytic inhibitor ICRF-193, Western blots did not show any apparent difference in the expression of topo IIα between the parental AA8 and the mutant EM9 cell lines (not shown).

3.2. Induction of endoreduplication by ICRF-193

As can be seen in Fig. 2, a characteristic feature observed in CHO cells after treatment with ICRF-193 at doses ranging from 0.025 to 1.0 μM was the presence of mitotic cells showing diplochromosomes, in good agreement with that reported earlier for the topo II inhibitors etoposide (VP-16) and mitoxantrone [4]. On the other hand, the presence of chromosomal

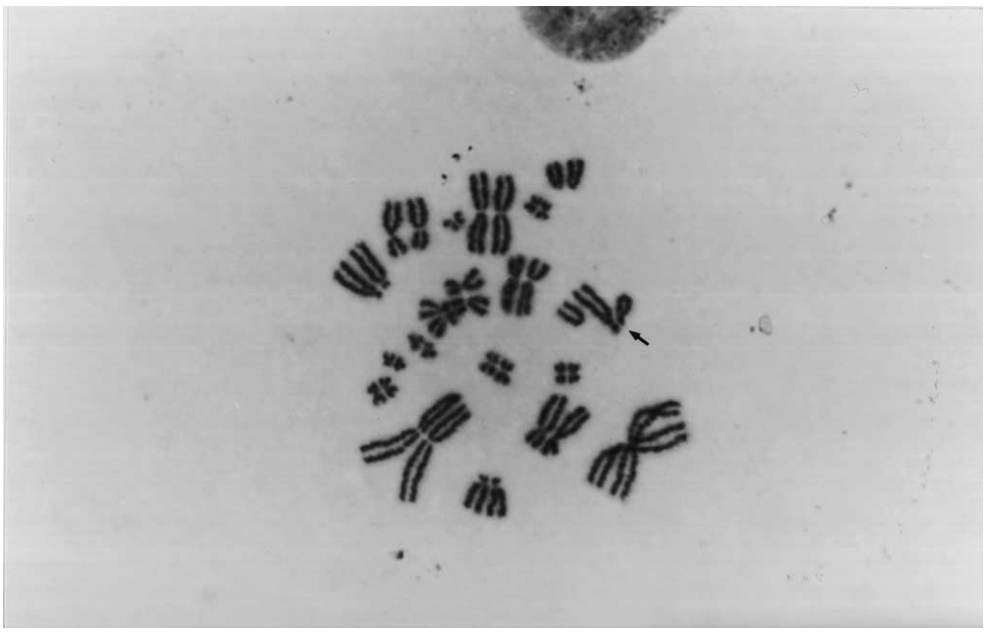

Fig. 2. EM9 endoreduplicated cell showing the characteristic diplochromosomes (made up of four chromatids) at metaphase after treatment with 0.05 μM ICRF-193. Note the chromosome aberration induced by the topo II catalytic inhibitor (arrow).

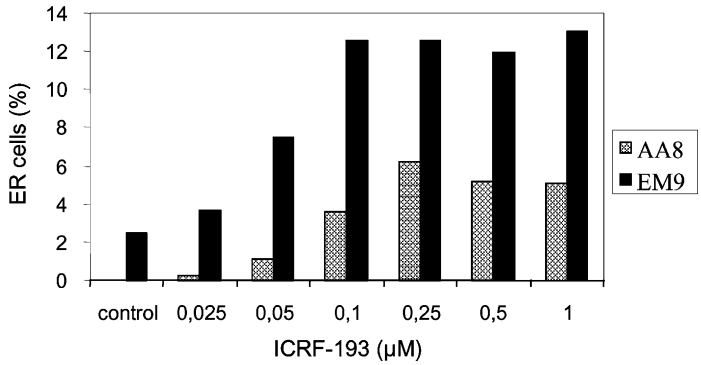

Fig. 3. Uneven spontaneous occurrence of endoreduplication and unequal response to treatment with the topo II catalytic inhibitor ICRF-193 in AA8 and EM9 cell lines. As can be seen, in absence of any treatment with the inhibitor, endoreduplicated (ER) AA8 cells were not observed, while approximately 2% of metaphases showing diplochromosomes were scored for EM9. Even taking into account this basal level in the EM9 mutant, an extremely high frequency of ER cells ($P < 0.001$; Student's t test), as compared with the parental AA8 cells, was a consistent observation for the whole range of doses tested. ICRF-193 doses of 2 μM and higher resulted in a very negative effect on cell division.

aberrations in endoreduplicated metaphases was also frequently observed (Fig. 2), as reported earlier by us for both the parental AA8 and the mutant EM9 cell lines [18]. For higher doses of ICRF-193, the low number of cells at mitosis made the accurate scoring of endoreduplicated cells impossible.

Fig. 3 shows the corresponding percentages of metaphases showing diplochromosomes observed in both AA8 and EM9 cell lines treated with a range of concentrations of ICRF-193. In addition to the presence of a relatively high number of endoreduplicated EM9 cells in non-treated controls, as reported [15], a consistent observation was that, for any given dose of the bisdioxopiperazine, the yield of endoreduplicated cells scored was higher in the CHO mutant EM9 than that observed in the parental AA8 cell line.

It is worth mentioning, on the other hand, that a steep drop in the number of mitotic cells as a whole was observed in cultures treated with doses of ICRF-193 of 2 μM and higher, recently found to be able to efficiently inducing chromosome damage [18].

4. Discussion

While many aspects of the molecular mechanism leading to endoreduplication, as well as in what concerns the behaviour of diplochromosomes remain as yet rather obscure, it is generally agreed that such a cytogenetical end-point is the indication of the failure of the normal chromosome separation process. On normal functioning this mechanism of segregation is fundamental for a proper distribution of genetic material during mitosis, after DNA replication during S-phase of the cell cycle.

An increasing body of evidence indicates that the conserved nuclear enzyme topo II should be necessary for chromosome segregation during mitosis, not only in yeast [19] but also in *Drosophila* [20], amphibia [21] and mammals [4,22–24]. In good agreement with this proposed role of the enzyme, if topo II function is blocked after chromosome condensation, the cells are arrested at metaphase and the chromatids fail to separate [19,25]. On the other hand, a role for topo II in meiotic chromosome condensation and segregation has also been reported in the yeast *Schizosaccharomyces pombe* [26] and mice [27,28], supporting a similar meiotic role for topo II as that played in mitosis for the segregation of sister chromatids after DNA replication. During meiosis, for separation in metaphase I of homologous chromosomes having one or more crossovers, topo II has to carry out the same task as in mitosis: separation of sister chromatids that are entangled because of meiotic DNA replication. Failure of this process should lead to arrest at the first meiotic division [27,28].

The reason why we have chosen the CHO DNA strand-break repair-defective mutant EM9 cell line for the investigation reported here is the uncommon feature that it presents as to a high rate of "spontaneous" endoreduplicated cells observed at metaphase, as compared to its parental line AA8 [15]. Based on the evidence reported above, a likely explanation is a failure of EM9 topo II to unravel daughter duplexes during either S, G_2 or mitosis, with a subsequent triggering of a new replication without any intervening anaphase [21–23].

Concerning this working hypothesis, in our opinion either a low level of topo II in the mutant EM9 as compared to AA8 parental cells, or a modified form of the enzyme which might result in a loss of activity to separate chromosomes should be responsible for the differences found between both cell lines in the yield of metaphases showing diplochromosomes even in absence of any treatment to induce endoreduplication. At this point, it is worth mentioning that in our analysis of the amount of topo II by immunoblotting in both cell lines, we have not observed any difference in the level of expression of the enzyme. This observation seems to rule out the first hypothesis, i.e. that EM9 should be characterized by a lower presence of topo II at the decisive moment of chromosome segregation. The possible expression of a modified form of the enzyme in the mutant EM9 cell line that might result in a lower rate of chromosome segregation at mitosis, however, remains open and will be discussed later on. Another possible explanation should be that differences in chromatin structure might be responsible for the occasional failure of EM9 to properly segregate daughter chromatids before cell division.

In order to further investigate the uncommon feature shown by EM9 cell line as to its apparently high basal level of endoreduplication, a topo II catalytic inhibitor, the bisdioxopiperazine ICRF-193 has been tested for comparison with its effectiveness in the parental line AA8.

The molecular mechanism of action of ICRF-193 on topo II has been recently elucidated. ICRF-193 acts on the nuclear enzyme activity without formation of any cleavable-complex [29], but catalytically it inhibits mammalian DNA topo II in a unique fashion. ICRF-193 stabilizes the closed clamp-form of the enzyme on DNA as a post-passage complex by inhibiting the intrinsic ATPase activity of the topo II, sequestering the enzyme from its normal turnover inside the cell [30].

Recently, we have reported that this process leads to chromosome damage, most likely through interference of such a "bulky" structure that results from the closed clamp conformation of topo II trapped on DNA with DNA replication or transcription machinery or even with DNA repair or chromatin assembly and disassembly [18,31]. This clastogenic effect appears as most likely responsible of the mitotic inhibition observed for the higher doses of ICRF-193, that made any scoring of endoreduplicated cells impossible.

Concerning daughter DNA strand unwinding by topo II for chromosome segregation, given our observation on an apparently similar amount of the enzyme in both EM9 and AA8 cells, a plausible explanation for the high yield of endoreduplication induced in EM9 by the topo II catalytic inhibitor tested by us should be that the mutant enzyme is more sensitive to ICRF-193 than that of the parental cell line. Nevertheless, possible diferences in chromatin structure between both cell lines that might result in the observed differences in both the spontaneous and bisdioxopiperazine-induced yield of endoreduplicated cells cannot at present be ruled out. In our opinion, this question deserves further investigation.

As to the fate of endoreduplicated cells arisen as a consequence of topo II catalytic inhibition, it has been reported that eventually they lose viability. Different observations seem to indicate that entry into mitosis without active topo II is lethal, even though the cells are capable of traversing further rounds of G_1- and S-phases [13]. It is noteworthy that, besides topo II, regulators of the cell cycle such as cyclin-dependent kinases seem to be involved in the complex and as yet poorly understood phenomenon of endoreduplication [32,33]. Another somewhat controversial point is, as regards the most sensitive period of the cell cycle for endoreduplication induction. While for some drugs the target for the induction of endoreduplication seems to be between S- and G_2-phases [34], other compounds have been reported to be metaphase inducers of endoreduplication [23,35].

In conclusion, it becomes apparent that the use of topo II catalytic inhibitors can be a new promising tool to unveil many mechanistic and physiological aspects of the complex and as yet rather obscure phenomenon of endoreduplication.

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