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# Halogen substitution of DNA protects from poisoning of topoisomerase II that results in DNA double-strand breaks

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ABSTRACT

DNA topoisomerase II (topo II), a fundamental nuclear enzyme, cleaves the double-stranded DNA molecule at preferred sequences within its recognition/binding sites. We have recently reported [F. Cortés, N. Pastor, S. Mateos, I. Domínguez, The nature of DNA plays a role in chromosome segregation: endoreduplication in halogen-substituted chromosomes, DNA Repair 2 (2003) 719–726] that when cells incorporate halogenated nucleosides analogues of thymidine into DNA, it interferes with normal chromosome segregation, as shown by an extraordinarily high yield of endoreduplication. The frequency of endoreduplicated cells paralleled the level of analogue substitution into DNA, lending support to the idea that thymidine analogue substitution into DNA is most likely responsible for the triggering of endoreduplication. Using the pulsed-field gel electrophoresis (PFGE) technique, we have now analyzed a possible protection provided by the incorporation of exogenous halogenated nucleosides against DNA breakage induced by the topo II poison *m*-AMSA. The result was that the different halogenated nucleosides were shown as able to protect DNA from double-strand breaks induced by *m*-AMSA depending such a protection upon the relative percent of incorporation of a given thymidine analogue into DNA. Our results clearly indicate that the presence of halogenated nucleosides in DNA diminishes the frequency of interaction of topo II with DNA and thus the frequency with which cleavage can occur.



## 1. Introduction

When cells incorporate halogenated nucleosides analogues of thymidine into DNA, it interferes with normal chromosome segregation, as shown by an extraordinarily high yield of endoreduplication [1]. Topological changes of DNA are necessary during many fundamental cellular processes such as replication, transcription and recombination. These changes take place through transient cleavage of the molecule, strand passing and religation carried out by DNA topoisomerases (topos), the key enzymes that catalyze the interconversion of topological isomers of DNA molecules (for a review, see [2]). Two main classes of topoisomerases have been described so far according to their catalytic mechanisms, namely type I topoisomerase, that breaks and rejoins one DNA strand at a time, and type II enzyme, that is able to do so with the two strands that make up duplex DNA. Given their molecular mechanisms of action, both types of enzymes are able to perform relaxation of supercoiled DNA which in turn relieves torsional tension generated during replication and transcription. Contrasting with topo I however, topo II is unique in that it is able to decatenate intertwined DNA molecules. This decatenating as well as unknotting activity of DNA topoisomerase II is essential for segregating replicated daughter chromosomes [3–9]. Besides, a lot of attention has been focused on DNA topo II after the discovery that the human enzyme is the target

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of a number of potent anticancer drugs of different chemical nature, and topo II inhibitors are well established in tumour chemotherapy [10,11].

Binding of the topo II homodimer to its double-stranded substrate is the first step of the enzyme's catalytic cycle of topoisomerization of DNA. This step does not seem to require any cofactor, but it is stimulated by the presence of divalent cations [12]. On the other hand, both nucleotide sequence and topology seem to play a role in the interaction topo II–DNA. While there is no report on high sequence specificity, topo II cleaves DNA at preferred sequences within its recognition/binding sites [13–16]. Besides the nucleotide sequence, the topological structure of DNA also influences the binding and cleavage by topo II, in such a way that it has been reported that supercoiled DNA shows as a much better substrate for enzyme interaction than relaxed molecules [13]. This behaviour of topo II explains its strong interaction with the supercoiled DNA substrate as well as its release of its relaxed reaction product when the topoisomerization cycle is complete.

Lending support to the hypothesis that a conserved nucleotide sequence is needed for topo II–DNA interaction leading to successful chromosome segregation during mitosis, we have recently reported [1] that efficient halogen substitution of DNA leads to a high yield of endoreduplication. Endoreduplication, a common symptom of disrupted cell cycle progression, more particularly progression through mitosis, becomes evident at metaphase by the presence of diplochromosomes, made up of four chromatids held together, instead of the normal two, as a consequence of two successive rounds of DNA replication without intervening mitosis, i.e. segregation of daughter chromatids [9,17]. These recent observations seem to agree with that reported earlier, showing 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 so-called "cleavable complex" [10,11] as well as those considered as true catalytic inhibitors [18] are able to induce endoreduplication [9,19,20] due to prevention of decatenation of replicated chromosomes by topo II with the subsequent failure to complete a normal mitosis. In the present investigation, we have examined the possible relative importance of DNA substitution by halogenated nucleosides analogues of thymidine for topo II interaction with DNA and its ability to form cleavable complexes for the enzyme to carry out its function.

## 2. Materials and methods

### 2.1. Cell culture

The Chinese hamster ovary fibroblast cell line AA8, purchased from American Type Culture Collection (ATCC, USA), was used in our experiments. Cells were grown as monolayers in McCoy's 5A medium (Bio Whittaker, USA) 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. Pulsed-field gel electrophoresis (PFGE) of DNA

Cells were treated for 24 h, i.e. roughly two cell cycles with the halogenated nucleosides analogues of thymidine, namely 5-chloro-2′-deoxyuridine (CldU), 5-bromo-2′-deoxyuridine (BrdU) or 5-iodo-2′-deoxyuridine (IdU) alone or in different combinations, depending upon the cell cycle on which a given analogue was present. Then, cells were treated for 3 h with the topo II poison 4-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA), at a dose of 2 μM, to induce DNA double-strand breaks through stabilization of topo II–DNA "cleavable complexes". Cells untreated, treated with halogenated nucleosides alone, or with *m*-AMSA alone served as controls. At the end of the treatments, cells were immediately embedded in agarose, and DNA double-strand breaks were analyzed by clamped homogeneous field (CHEF) gel electrophoresis as reported elsewhere [21]. The procedure was as follows: exponential cells were collected using a cell scraper, washed twice in cold phosphate-buffered saline (PBS) and counted in PBS. They were mixed with low-melting temperature agarose (LMP-agarose, Sigma) at $4 \times 10^6$ cells/ml. The suspension was pippeted into plug moulds (250 μl, Bio-Rad) and kept at 4 °C for 30 min to allow the agarose to set. Subsequently, plugs were transferred to three volumes of lysis solution (50 mM EDTA, 2% sarkosyl and 0.5 mg/ml proteinase k), maintained on ice for 1 h to prevent any repair occurring during diffusion of the lysis solution and then incubated at 37 °C for 24 h. Before electrophoresis, the plugs were washed twice in PBS and cut into halves corresponding to about $2 \times 10^4$ cells. These samples were inserted into 0.6 cm × 0.5 cm × 0.1 cm wells of a precast 0.8% agarose gel in 0.5× TBE buffer (50 mM Tris, 50 mM borate, 0.1 mM EDTA; pH 8.4). *Saccharomyces cerevisiae* yeast chromosomes were used as DNA size standards in each gel. The wells were sealed with 0.8% agarose. Electrophoresis was carried out using a CHEF-DRII system (Bio-Rad). The gels were electrophoresed at 45 V (1.3 V/cm) for 96 h with a switch time of 60 min. Electrophoresis buffer was 0.5× TBE. Buffer temperature was maintained at 14 °C by circulation through a cooling bath. Following electrophoresis the gels were placed in 200 ml of electrophoresis buffer with 0.5 μg/ml of ethidium bromide to stain the DNA. Finally, gels were photographed under UV illumination and densitometrically analyzed using the software programme PCBAS Version 2.08. DNA damage was measured as the percent of DNA migration from the well: % DNA migration = [optical density in lane/total optical density on the lane and well] × 100. Statistical analysis for significance was used (Student's *t*-test).

Thymidine analogues substitution into DNA was always assessed by the fluorescent plus Giemsa (FPG) staining method as reported elsewhere [22,23]. All the experiments were carried out in triplicate.

### 2.3. Preparation of nuclear extracts

Topo II activity in nuclear extracts from AA8 cells was obtained as described by Heartlein et al. [24]. Approximately $1 \times 10^7$ 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 × *g* (Eppendorf centrifuge),

for 5 min at 4 °C. Nuclei were then washed in 1 ml of nucleus wash buffer ($5 \times 10^{-3}$ M potassium phosphate buffer, pH 7.5, $10^{-3}$ M phenylmethyl sulfonyl fluoride (PMSF), $10^{-3}$ M β-mercaptoethanol and $0.5 \times 10^{-3}$ M dithiothreitol (DTT)). The nuclei were then pelleted as described above and resuspended in 50 μl of nucleus wash buffer, and 50 μl of $4 \times 10^{-3}$ M EDTA was added. Following incubation at 0 °C for 15 min, the nuclei were lysed by adding 100 μl of 2 M NaCl, $20 \times 10^{-3}$ M Tris–HCl, pH 7.5, $10^{-2}$ M β-mercaptoethanol and $10^{-3}$ M PMSF. Following a 15 min incubation at 0 °C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, $50 \times 10^{-3}$ M Tris–HCl, pH 7.5, $10^{-2}$ M β-mercaptoethanol and $10^{-3}$ M 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 DNA/protein pellet was removed from the extract to a new tube and the same amount of extraction buffer was added as in the previous step. Re-extraction to determine the fraction of DNA-bound topo II activity was then done with 0.9 M NaCl (200 μl/ml extraction buffer). The suspension was incubated for 30 min at 0 °C and the supernatant from a 20 min centrifugation at $11{,}200 \times g$ at 4 °C was then colleted. Total protein concentration in each extract [25] was determined in a Beckman DU-64 spectrophotometer by the Bio-Rad protein assay (Bio-Rad Laboratories) and the extracts were kept at −80 °C for no longer than a month.

### 2.4. *Topoisomerase II activity in nuclear extracts*

Topo II activity in nuclear extracts from either untreated cells, cells treated with *m*-AMSA alone, or halogen-substituted before treatment with the topo II poison, was assayed using an assay kit (TopoGen, Columbus OH, USA) based upon decatenation of kinetoplast DNA (kDNA). Reaction products were resolved using agarose gel electrophoresis of DNA. After incubation, 40 min 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.

## 3. Results and discussion

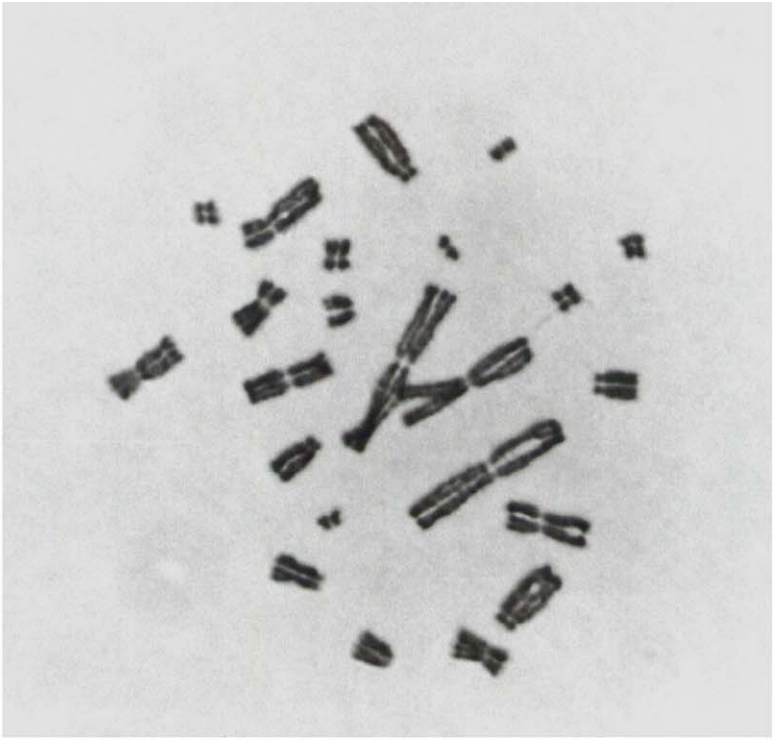
Both topo II "poisons" [10,11] and catalytic inhibitors [18] are able to induce endoreduplication [9,19,20]. We have recently reported [1] that incorporation of halogenated pyrimidines into DNA in place of thymidine also induces endoreduplication (Fig. 1a). According to our working hypothesis, this is most likely due to a loss of recognition/binding of the enzyme to its DNA sequence. To test this, we have assessed topo II function as its ability to covalently bind to DNA as a first necessary step for the stabilization of the cleavable complex as a consequence of poisoning of this otherwise fleeting intermediate [10] (Fig. 1b and c). First, we analyzed the possible influence of the halogenated nucleosides CldU, IdU and BrdU after their incorporation into DNA for thymidine for two cell cycles, on DNA damage induced by a 3 h treatment with the topo II poison *m*-AMSA. As can be seen in Fig. 1b, all the halogen-substituted nucleosides incorporated in DNA were shown as able to protect the molecule against the production of double-strand breaks induced by amsacrine in a percent of substitution-dependent fashion (Fig. 1b and c).

To determine whether substitution of the halogenated nucleoside for thymidine for two consecutive S-periods, i.e. 75% of DNA strands halogen-substituted (in 50% of the duplexes, in both parental and daughter strands; in the other 50% of the duplexes, only in daughter strands) was necessary for protection of DNA from topo II poisoning and cleavable complex stabilization, we treated the cells for just one cell cycle with CldU, as a representative halogenated nucleoside (all of the duplexes halogen-substituted, but only in daughter DNA) and the result was that this alternative experimental schedule also yielded a significant protective effect (Fig. 2). It is noteworthy that after 100% CldU substitution for just one cell cycle, a significantly higher protection (higher than 70%) was observed as compared with that found after incorporation of the halogenated pyrimidine for two cell cycles (only about 60% protection). Since in this latter case half of the duplexes are bisubstituted and the rest monosubstituted with CldU, while all of them are monosubstituted after only one S-period of halogen incorporation, the higher protection observed following this latter experimental design seems somewhat paradoxical. Concerning this, although an explanation is not at hand, in our opinion a likely possibility should be that after two cell cycles a number of misincorporated halogenated pyrimidines might be removed from DNA by an excision repair pathway that operates on them.

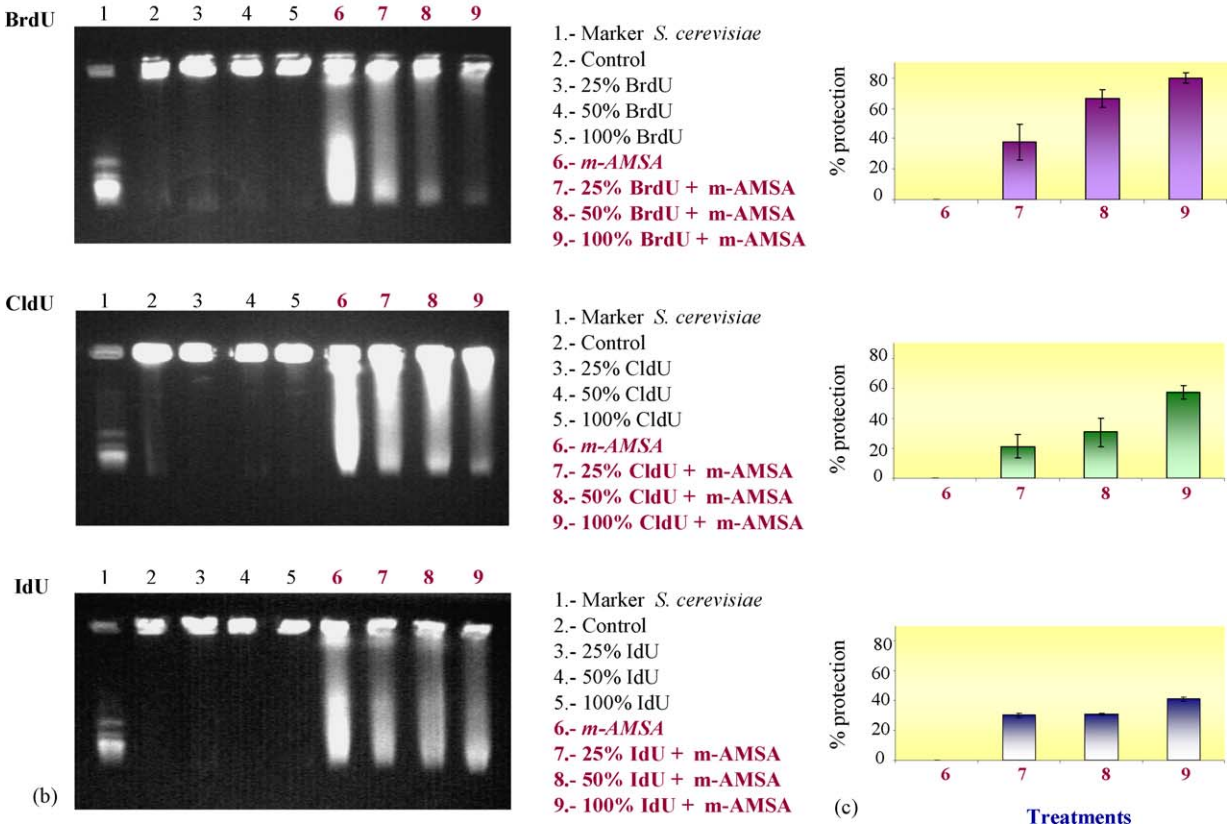
Next, we examined the possible differences in the protection provided by different combinations of halogenated nucleosides, namely CldU and BrdU, depending upon the S-period (either the first or the second) in which the incorporation into DNA takes place. As expected from their previously observed particular protective effectiveness (Fig. 3a, c and d) no significant difference was found for a combined experimental schedule regardless of the cell cycle in which DNA was fully substituted (100%) with any of the halogenated nucleosides (Fig. 3a).

Focusing on CldU, on the other hand, a significant difference in the protection from DNA breakage accomplished, however, was observed when the degree of CldU incorporation into DNA in either the first or the second S-period was largely different. Rates of CldU substitution for thymidine as extreme as 10 and 100% were chosen, and Fig. 3b shows the nature of DNA, i.e. fully CldU-substituted, partly substituted or unsubstituted, according to the different experimental designs carried out. Comparative results provided by PFGE are shown in Fig. 3c and d. As can be seen, a protective effect of about four-fold (60.9% of protection from double-strand breaks) was observed when CldU substitution during the last period before *m*-AMSA treatment was roughly 100%, as compared with that found when DNA was only slightly substituted (10%). Lending further support to the hypothesis that modifications in DNA sequence lead to malfunction of topo II, we have also observed a sound correlation between the above results on DNA protection by halogenated pyrimidines against topo II-mediated *m*-AMSA breakage and that reported earlier by us using similar experimental conditions [1] on the induction of endoreduplication as an end-point that measures a possible failure of the enzyme performance in chromosome segregation during mitosis.

The results presented above, as well as those on induction of endoreduplication by halogenated pyrimidines [1] and by the demethylating agent 5-azacytidine as well [21] recently reported by our research group, suggest that the preservation of a normal DNA sequence plays a major role for topo II function. As a matter of fact, an interesting observation was that the fraction of topo II activity bound to DNA [26] trapped in the cleavable complexes after treatment with *m*-AMSA, diminishes when DNA is fully substituted for just one S-period with any of the three halogenated nucleosides tested in our study (Fig. 4). In our opinion, this observation lends a strong support to the hypothesis on the lack of recognition by topo



(a)




(b)

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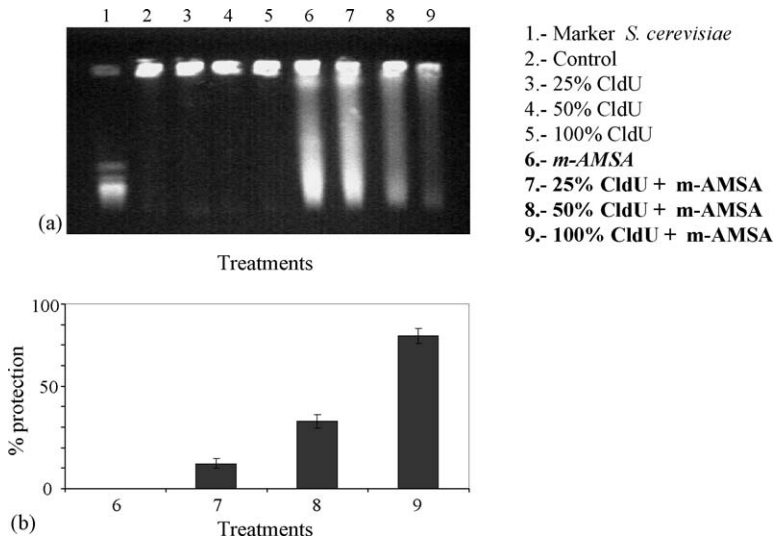


**Fig. 2 – CldU substitution into DNA for one cell cycle is enough to provide protection from the production of DNA double-strand breaks by the topo II poison *m*-AMSA. (a) Increasing CldU incorporation (25, 50 and 100%) into DNA for thymidine and assessment of *m*-AMSA-induced DNA double-strand breaks using PFGE. (b) Histogram showing the densitometric measurements of the particular reduction in the total amount of DNA migrating from the wells as observed by PFGE (see above). As can be seen, the level of CldU incorporation into DNA parallels the degree of protection observed against DNA double-strand breaks induced by *m*-AMSA. It is noteworthy that more than 70% of the total yield of DNA double-strand breaks was reduced when DNA was previously fully substituted with CldU (see lane 9).**

II of DNA when it is halogen-substituted for thymidine. It is widely accepted that topo II catalytic activity is necessary for segregating replicated daughter chromatids before anaphase [3,27,28]. Indeed, we have recently reported a very efficient induction of endoreduplication by the topo II specific inhibitor ICRF-193, a bis-dioxopiperazine [20]. The interest of investigations such as the present stems from the fact that even though it is generally agreed upon that nucleotide sequence plays a role, the rules that determine the nucleic acid specificity of topo II are as yet far from being completely elucidated. It has been reported that topo II cleaves DNA at preferred sequences within its recognition/binding sites, but there is no report on high specificity [13–16]. In order to study the sequence specificity of double-strand DNA cleavage by *Drosophila* topo II, the frequencies of the nucleotides and dinucleotides in the region near the site of phosphodiester bond breakage were analyzed [29] and revealed a non-random distribution. The consensus sequence derived was 5′GT.A/TAY↓ATT.AT..G3′, where a dot means no preferred nucleotide and Y stands for pyrimidine [29]. On the other hand, DNase I footprint analysis has revealed that *Drosophila* topo II can protect a region in both strands of the duplex DNA, with the cleavage site located near the centre of the protected region [15], and it has been proposed that the strong DNA cleavage sites of *Drosophila* topo II [29]

**Fig. 1 – Endoreduplication and protection provided by halogen substitution in DNA in place of thymidine for two consecutive S-periods against DNA-strand breaks induced by the topo II poison *m*-AMSA. (a) Endoreduplicated Chinese hamster cell at metaphase after BrdU substitution in DNA for thymidine. Note the characteristic diplochromosomes, made up of four chromatids instead of the normal two. This end-point is used for the assessment of malfunction in chromosome segregation leading to genetic instability and often to cell death. (b) Assessment of the possible protection from *m*-AMSA induced DNA double-strand breaks by means of PFGE. The covalent "cleavable complex" DNA–topo II are stabilized by *m*-AMSA. Collision of moving replication fork with such a stabilized ternary complex DNA–topo II-poison leads to DNA double-strand breakage. AA8 Chinese hamster ovary fibroblasts were given three levels (25, 50 and 100%) of either BrdU, CldU or IdU for two cell cycles and then treated with the topo II poison *m*-AMSA. To achieve the desired degree of substitution of thymidine with halogenated nucleosides, deoxythymidine was present simultaneously with the halogenated pyrimidines in the presence of the DNA synthesis inhibitor 5-fluordeoxyuridine. One hundred percent substitution means that every thymidine in DNA has been replaced, and so on. A representative PFGE gel of three independent experiments is shown in each case. (c) Values from densitometric analysis expressed as percent of DNA migration in PFGE gels is shown above, indicative of DNA double-strand breaks produced. Percentage of protection as compared to the migration observed for *m*-AMSA alone (no protection whatsoever) was measured for each combined treatment halogenated nucleoside + *m*-AMSA. Each bar represents the mean of three independent experiments ± S.D. Differences were in all cases statistically significant ($P < 0.001$, according to Student's *t*-test).**

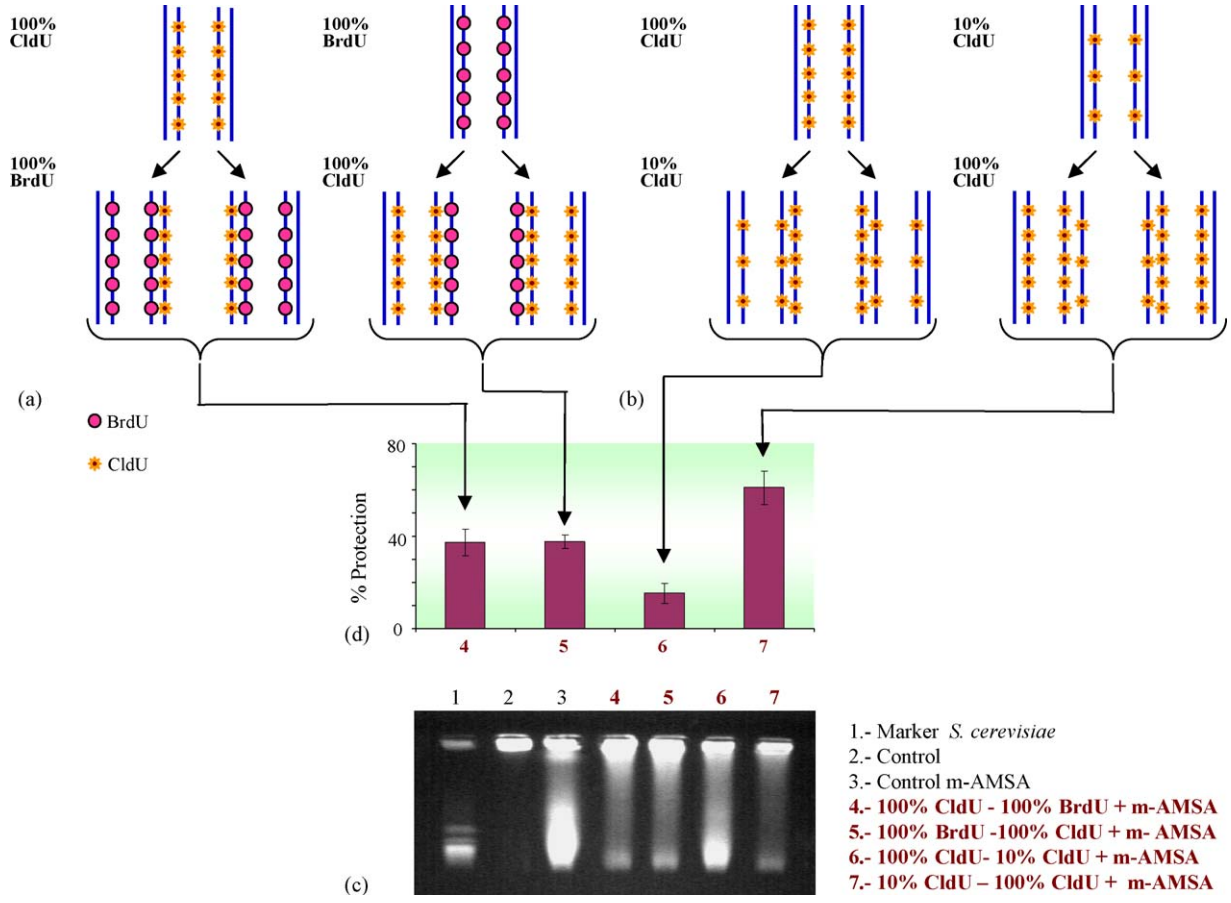


**Fig. 3 – Comparative analysis of the relative importance of the nature of the particular halogen as well as the degree of halogen substitution into DNA during either the first or the second S-period. (a) Schematic diagrams showing the substitution of DNA strands with either CldU or BrdU throughout two successive S-periods as well as their segregation into chromatids. (b) The same when only CldU is substituted for thymidine for two cell cycles, but the degree of substitution varies extremely (10 and 100% substitution in either the first or the second S-period). (c) Representative PFGE gel showing the respective protection from the topo II poison *m*-AMSA-induced DNA double-strand breaks observed in each case. (d) Densitometric evaluation of the PFGE gels (in triplicate) to assess the degree of protection achieved for each combined treatment as described above. As can be seen, while the combinations of 100% BrdU and 100% CldU provided a similarly good protection, regardless of their particular presence in either the first or the second S-period, a dramatic difference was the outcome for 10 and 100% CldU combinations.**

likely correspond to specific DNA-binding sites of the enzyme [13,30]. Furthermore, the interaction between calf thymus topo II and DNA was also characterized by means of a transcription assay [31] and it was concluded that topo II binds to a region of DNA located symmetrically around the enzyme-mediated cleavage site.

We have demonstrated unequivocally the breakdown of the catalytic cycle of DNA breakage and rejoining of topo II as a result of a lack of recognition of specific thymine-rich DNA sequences by the enzyme, as shown by a loss of cleavable complex formation in halogen-substituted DNA. This in turn results in an unsuccessful DNA segregation of fully replicated chromosomes that eventually triggers an endoreduplication cycle [1,21], a rare event that begins with an aberrant mitosis without a proper anaphase. The subsequent replication of non-split chromosomes leads to the final outcome as the characteristic diplochromosomes, made up of four chromatids instead of the normal two, show up in the next mitosis [9,17].

In our opinion, even though the possible involvement of other proteins cannot be ruled out at present, our observations seem to favor the likely hypothesis that the nature of DNA might play a role for the recognition/binding of topo II and its subsequent cleavage of the fully replicated molecule for chromosome segregation. It has been reported that eukaryotic topo II preferentially cleaves alternating purine–pyrimidine repeats within the consensus sequence, and additionally, GT, AC and AT repeats were better substrates for cleavage than GC repeats [14,15]. Furthermore, the distribution of DNA cleavage sites induced by topo II in the presence or absence of enzyme poisons were mapped in the simian virus 40 genome [16] and the finding was that strong sites tended to occur within A/T runs such as those that have been associated with binding to the nuclear scaffold [16]. Our present results seem to support these observations on the relative importance of thymine for topo II recognition/binding to DNA.

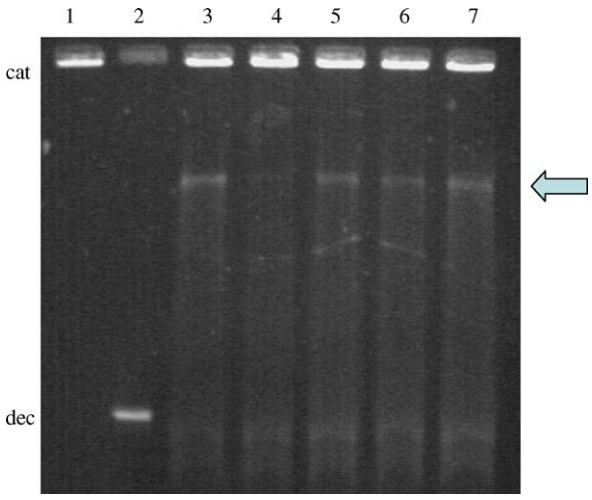


**Fig. 4 – Increased topo II activity bound to DNA as a consequence of trapping of the enzyme in the cleavable complex topo II–DNA–*m*-AMSA and influence of halogen-substitution before treatment with the topo II poison as shown by DNA gel electrophoresis. Lane 1: marker catenated (cat) DNA; lane 2: marker decatenated (dec) DNA; lane 3: control non-treated cells; lane 4: cells treated for 3 h with 2 μM *m*-AMSA alone; lanes 5–7: cells substituted with CldU, BrdU and IdU, respectively, for one cell cycle before treatment with *m*-AMSA. Note that the topo II activity bound to DNA extractable is low in this cell line (see lane 3), as shown by the presence of a band near the well (arrow). As a result of *m*-AMSA trapping of the enzyme in the cleavable complexes, a higher activity appears as responsible of the absence of this band that clearly shows up when the DNA has been halogen substituted.**

As a consequence of their ease to incorporate into DNA as thymidine analogues, halogenated nucleosides have been reported to be potentially mutagenic [32,33] and to induce sister chromatid exchanges [34,35] as well as chromosomal aberrations [36]. We chose three of them, namely CldU, BrdU and IdU to see whether or not there was an effect on the normal relationship DNA–topo II that in turn might affect chromosome segregation. Our observation was that all the halogenated nucleosides tested were able to protect DNA from strand breakage induced by poisoning of topo II, in such a way that the relative protection paralleled the relative percentage established concerning the halogenated pyrimidine:deoxythymidine incorporation into DNA. Taken as a whole, our results seem to support the idea that the presence of anomalous bases such as halogenated pyrimidines or demethylated cytosine [21] in DNA results in a defective function of topo II as a whole, while malfunction in chromosome segregation eventually leads to aberrant mitosis and the subsequent endoreduplication [1,20]. The present evidence that the nuclear enzyme topo II plays a major role in the separation of fully replicated double-stranded DNA provides support to the idea that also in mammalian cells, even though temperature-sensitive mutants such as those used in yeast [3] are not available, the enzyme is of paramount importance during mitosis.

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