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DNA strand breaks induced by the anti-topoisomerase II bis-dioxopiperazine ICRF-193

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Abstract

The bis-dioxopiperazine ICRF-193 has long time been considered as a pure topoisomerase II catalytic inhibitor able to exert its inhibitory effect on the enzyme without stabilization of the so-called cleavable complex formed by DNA covalently bound to topoisomerase II. In recent years, however, this concept has been challenged, as a number of reports have shown that ICRF-193 really "poisons" the enzyme, most likely through a different mechanism from that shown by the classical topoisomerase II poisons used in cancer chemotherapy. In the present investigation, we have carried out a study of the capacity of ICRF-193 to induce DNA strand breaks, as classical poisons do, in cultured V79 and *irs-2* Chinese hamster lung fibroblasts using the comet assay and pulsed-field gel electrophoresis (PFGE). Our results clearly show that ICRF-193 readily induces breakage in DNA through a mechanism as yet poorly understood.
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Keywords: ICRF-193; Topoisomerase II; Cytotoxicity; DNA damage

1. Introduction

DNA topoisomerases are evolutionary conserved nuclear enzymes involved in a number of nuclear processes, including replication, transcription and recombination. Topoisomerases relax superhelical tension catalyzing the unlinking of DNA strands by making transient DNA strand breaks and allowing another DNA to pass through these breaks. There are two classes of topoisomerases according to their catalytic mechanisms. The type I topoisomerases (topo I) cleave one strand of duplex DNA and do not require energy for topoisomerization. On the other hand, type II topoisomerases (topo II) use a complex sequential mechanism of ATP hydrolysis to catalyze the cleavage of both strands of the DNA molecule and transport an intact DNA duplex through a transient double-stranded break [1,2].

In cancer chemotherapy topo II is a major target for a variety of anticancer drugs. According to their mode of action, these drugs have been divided into two classes. Drugs that stabilize the protein-linked DNA intermediate termed the cleavable complex and produce a double-strand break (DSB) through this complex are referred to as DNA topoisomerase poisons [3] and belong to the first class. The other class, which includes bis-dioxopiperazines (ICRF-187, ICRF-193, etc.), fostriecin, aclarubicin and merbarone, inhibits topo II by different mechanisms apparently without forming a cleavable complex. These latter compounds are classified as "true" catalytic inhibitors because, according to several reports, no DNA lesions are

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formed [2]. Among these drugs, ICRF-193 has a unique property in that it acts through stabilization of the circular clamp conformation of the DNA–topo II-complex, thus preventing strand passage [4].

Several X-ray-sensitive cell lines are hypersensitive to DNA–topo II inhibitors such as etoposide, which stabilize topo II–DNA cleavable complexes and thus concomitantly induce double-strand breaks [5]. This hypersensitivity has generally been ascribed to a deficiency of these mutant cell lines in DSB repair [6,7]. In contrast to etoposide, ICRF-193 apparently inhibits DNA–topo II activity without inducing any DSB thus allowing to separate the effects of DNA–topo II inhibition from those due to the introduction of DSB [8,9]. However, data can be found in the literature that point to the potential of this drug to act as a novel type of topo II poison [10]. Furthermore, recently, Huang et al. [11], have shown that ICRF-193 causes both topo II–DNA cross-links and specific topo II-mediated DNA cleavages.

Since controversial data have been reported about the ability of ICRF-193 to produce DNA damage, the purpose of the present study was to evaluate any DNA-damaging activity of ICRF-193 using two cultured Chinese hamster lung fibroblast cell lines, the repair proficient parental V79 and its radiosensitive mutant *irs-2*. This latter cell line shows a defect similar to that found in the human syndrome ataxia telangiectasia (AT), i.e. a radioresistant DNA synthesis [12]. Two different protocols, the comet assay and the pulsed-field gel electrophoresis (PFGE) technique were used. The comet assay allows the detection of single-strand break (SSB) and double-strand break and, with the method at high pH employed here, to visualize alkali-labile sites in individual cells. PFGE has been employed to measure DSB, most important lesions in terms of cytotoxicity. The results obtained are consistent with the induction of DSB and are discussed taking into account the known or assumed mechanisms of topo II inhibition by this bis-dioxopiperazines.

2. Materials and methods

2.1. Chemicals

The topo II inhibitors *m*-AMSA (NSC-249992) and ICRF-193 were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD (USA), and from BIOMOL Feinchemikalien GmbH, Hamburg (Germany), respectively. The DNA synthesis inhibitor aphidicolin (APH) was purchased from Sigma Chemical Co., St. Louis, MO (USA).

2.2. Cells and culture conditions

The parental Chinese hamster cell line V79 was purchased from the American Type Culture Collection (ATCC), USA. The mutant *irs2* was kindly provided by Dr. John Thacker (Medical Research Council, Harwell, UK). Cells were routinely maintained as monolayers in Minimum Essential Medium (Gibco BRL) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and the antibiotics penicillin (50 units ml^{-1}) and streptomycin (50 $\mu g\,ml^{-1}$). 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.

2.3. Cell viability

V79 and *irs2* cells in exponential growth phase were harvested using Trypsin–EDTA (Gibco BRL), and resuspended in medium. They were seeded at 5×10^3 cells/100 μl in 96-well microtitre plates (Nunc) and allowed 24 h to attach. Then, they were incubated further for 48 h in the presence of the DNA topoisomerase II inhibitor ICRF-193. The concentration range tested (0.005–10 μM) was prepared in tissue culture medium from 1 mM ICRF-193 stock solution.

Following the recommendations of the National Cancer Institute (USA), the analysis of cytotoxic effects induced by ICRF-193 was determined using a sulforhodamine B (SRB) assay as described previously [13,14]. Briefly, 50 μl per well of 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 then stained with 100 μl per well of 0.4% SRB 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

solubilized in 100 μl per well of 10 mM unbuffered Tris base by shaking. The optical density was read at 492 nm using a microtitre plate reader (ELISA). Each type of experiment was independently performed in triplicate.

2.4. Preparation of nuclear extracts

Exponentially growing V79 and *irs2* cells were incubated for 3 h in the presence of different concentrations (0.5, 1, 2.5, 5, and 10 μM) of the topo II inhibitor ICRF-193. 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 followed was basically that described by Heartlein et al. [15]. 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 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 lyzed 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 was added. The suspension was incubated for a further 40 min period at 0 °C. Then the supernatant from a 30 min centrifugation at 12,500 rpm at 4 °C was collected. Total protein concentration in each extract was determined in a Beckman DU-64 spectrophotometer by the Bradford protein assay [16]. Extracts were kept for no longer than a month at −80 °C.

2.5. 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). One hundred nanograms of nuclear extract protein from each cell line was incubated with different doses (0.5, 1, 2.5, 5, and 10 μM) of ICRF-193. 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 $\mu g\,ml^{-1}$ ethidium bromide, destained (30 min) in distilled water and photographed using a standard photodyne set.

2.6. Comet assay

V79 and *irs2* cells were treated for 3 h with different concentrations (1, 2.5, 5, and 10 μM) of ICRF-193. Positive controls of both cell lines were obtained after irradiation of exponentially growing cells with 5 Gy X-rays using a X-ray machine (Philips MG 103/2.25 system, Germany, 100 kVp, 15 mA, dose rate 1 $Gy\,min^{-1}$). To determine the inicial DNA damage cells were irradiated on ice.

The assay was basically performed according to the original protocol of Singh et al. [17]. Briefly, the standard slides were immersed vertically in 1% normal melting agarose (NMA) at 55 °C and left vertically to allow the agarose to solidify. The slides were then kept at 4 °C until use.

Approximately 10,000 cells were mixed with 85 μl of low-melting agarose (LMA; 0.7% in PBS) (FMC) at 37 °C and, the cell suspension was rapidly pipetted onto the first agarose layer, spread using a coverslip and kept at 4 °C for 8 min for the LMA to solidify. The coverslips were then removed, and a third layer of 100 μl LMA (0.7%) at 37 °C was added, covered with a coverslip, and again allowed to solidify at 4 °C for 8 min. After the top layer of agarose was solidified, the slides were immersed in a chilled lysis solution made up of 2.5 M NaCl, 0.1 M Na_2EDTA, 10^{-2} M Tris–HCl, 1% sodium sarcosinate, pH 10, with 1% Triton X-100 and 10% DMSO added just before use. They were kept at 4 °C in the dark for at least 1 h to lyse the cells and to allow DNA unfolding.

The slides were removed from the lysis solution, drained and placed on a horizontal gel electrophoresis unit, side by side. The tank was filled with chilled fresh alkaline solution (10^{-3} M Na_2 EDTA, 0.3 M NaOH) at 4 °C and pH 12.8, in order to detect double- and single-strand breaks as well as alkali-labile sites [18].

Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of DNA. Electrophoresis was carried out at low temperature (4 °C) for 20 min at 1.6 V cm^{-1} and 300 mA. In order to prevent additional DNA damage, all the steps described above were conducted under yellow light or in the dark.

After electrophoresis, slides were gently washed in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove alkali and detergent, and stained with 50 μl DAPI (5 μg ml^{-1}) in Vectashield (mounting medium for fluorescence H-1000, Vector Laboratories, USA).

DNA of individual cells was viewed using an epifluorescence microscope OLYMPUS Vanox AHBT3, with an excitation filter of 550 nm and barrier filter of 590 nm, connected to a CCD camera and a pentium computer. Images of 50 randomly selected cells were captured by digitization from each sample. They were examined automatically using an image analysis CASys software (Synoptics Ltd., image processing systems, UK) [19]. The measure of damage was tail moment, which is an integral of the distance and amount of DNA that has migrated out of the comet "head". An increase of DNA tail moments over the control is a measure of DNA damage.

2.7. Pulsed-field gel electrophoresis (PFGE) of DNA

Cells were treated for 3 h with different doses of ICRF-193 ranging from 1 to 10 μM, or 2 μM *m*-AMSA. Treatment with 10 μM of the DNA synthesis inhibitor APH was started 5 min before ICRF-193 or *m*-AMSA treatments, continued throughout the ensuing 3 h, and ended at the same time as the topoisomerases inhibitors. At the end of the drug treatments, cells were immediately embedded in agarose, and DNA double-strand breaks were analyzed by clamped homogeneous field (CHEF) gel electrophoresis.

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 4×10^6 cells ml^{-1}. 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 (0.5 mol dm^{-3} EDTA, 2% sarkosyl and 0.5 mg ml^{-1} 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 2×10^4 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 (0.05 mol dm^{-3} Tris, 0.05 mol dm^{-3} borate, 0.1 mmol dm^{-3} EDTA; pH 8.4). *Sacharomyces 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^{-1}) 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^{-1} of ethidium bromide to stain the DNA. Finally, gels were photographed under UV illumination and densitometrically analyzed using the software program PCBAS version 2.08. DNA damage was measured as the percent of DNA migration from the well: % DNA migration = [optic density in lane/ total optic density of the lane and well] × 100.

3. Results

3.1. Cytotoxicity of ICRF-193

These studies were initiated in order to determine the ability of ICRF-193 to produce per se DNA damage as a result of topo II inhibition. Also, we wanted to know whether the radiosensitive mutant *irs-2* would prove to be sensitive to ICRF-193 when compared to the parental line V79. First, the cytotoxicity of ICRF-193 was determined by means of the SRB assay. Fig. 1 shows the results obtained after the treatment of the parental cell line V79 and its radiosensitive derivative *irs-2* with ICRF-193 at different concentrations. As can be seen, in general, a similar decrease in cell viability was observed in both cell strains for doses up to 2.5 μM. However, for the two highest doses used in this study, i.e. 5 and 10 μM,

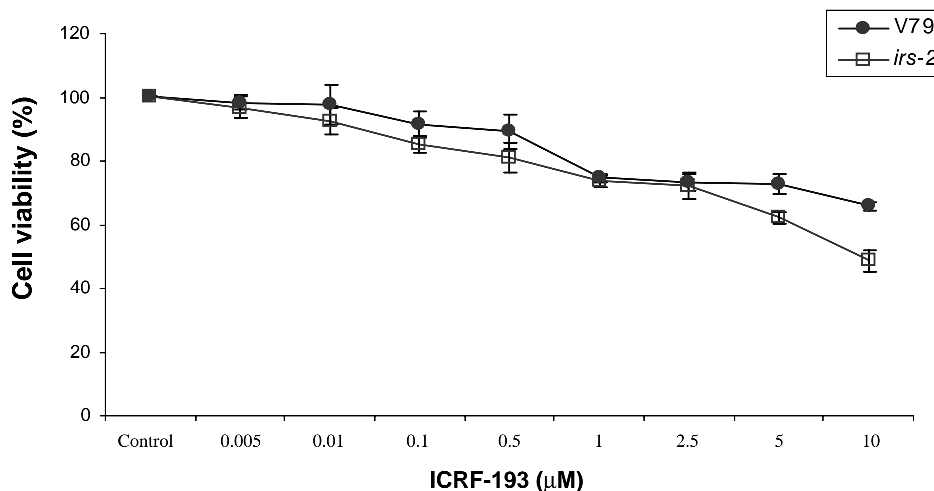

Fig. 1. Comparative effects of different concentrations of the anti-topo II ICRF-193 to suppress cell viability in the parental cell line V79 and its radiosensitive cell mutant *irs-2* as shown by the SRB assay. Bars indicate standard deviation from three independent experiments. Statistical analysis (Student's *t*-test) shows a significant difference ($P = 0.02$) between both cell lines only for the two highest doses of ICRF-193 employed.

a higher sensitivity was observed in the radiosensitive mutant *irs-2*.

3.2. Effect of ICRF-193 on topo II catalytic activity

Inhibition of topo II activity by ICRF-193 was also analyzed as a loss in the capacity to decatenate double-stranded catenated kinetoplast DNA (kDNA). Fig. 2 shows that in the absence of drug, topo II activity in nuclear extracts from V79 (Fig. 2A) and *irs-2* cells (Fig. 2B) was able to efficiently release catenated k-DNA. Also, Fig. 2A and B shows that increasing concentrations of ICRF-193 produce an inhibition of topo II catalytic activity in both cell lines, as indicated by a similar increase in the amount of catenated substrate remaining in the wells. However, subtle differences have been found between both cell lines. For example, partial inhibition of the enzyme was observed in *irs-2* at the lowest ICRF-193 concentration, whereas there was absence of inhibition in the parental cell line. However, in both cell lines, a similar intermediate inhibition was seen at the higher concentrations and total inhibition at 10 μM ICRF-193. Therefore, in accord with the inhibitory effects of ICRF-193 on cell viability (Fig. 1), these results indicate that this drug can efficiently inhibit topo II catalytic activity in both cell lines as well.

3.3. DNA damage induced by ICRF-193

The induction of DNA damage by ICRF-193 was first investigated using the alkaline single cell gel electrophoresis (SCGE) or "comet assay" which provides a measure of both SSB and DSB as well as alkali-labile sites in DNA. Following a 3 h treatment at 37 °C with ICRF-193, DNA damage was observed in cell lines V79 and *irs-2* at concentrations ranging from 1 to 10 μM (Fig. 3A and B), respectively. Fig. 3 also shows that the radiosensitive mutant *irs-2* is more sensitive to DNA breakage induced by ICRF-193 than its parental line V79 as evidenced by the higher tail moments for given concentrations (compare Fig. 3A and B).

The DNA-damaging activity of ICRF-193 was confirmed using the pulsed-field gel electrophoresis technique, which specifically allows to detect DSB in DNA. Fig. 4A and B shows the total amount of double-stranded DNA released from the plugs of Chinese hamster V79 and *irs-2*, respectively, after 3 h incubation at increasing concentrations of ICRF-193. There is a concentration-dependent production of DSB. Again, the extent of the DNA damage produced

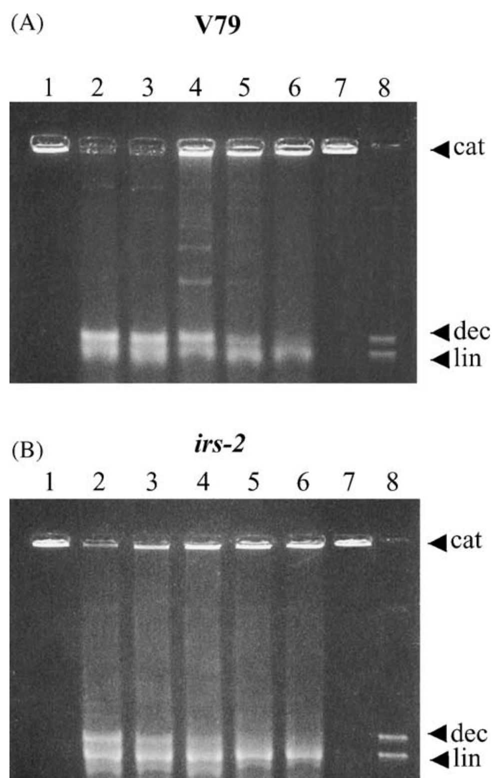

Fig. 2. Effectiveness of different doses of ICRF-193, ranging from 0.5 to 10 μM to inhibit the topo II catalytic activity. Nuclear extracts from V79 (A) and *irs-2* cells (B) were obtained as described in material and methods and their ability to decatenate catenated kinetoplast DNA was assayed by DNA gel electrophoresis. Lane 1: marker catenated (cat) kinetoplast DNA; lane 2: control non-treated with ICRF-193; lanes 3–7 treated with increasing concentrations of ICRF-193 (0.5, 1, 2.5, 5, and 10 μM, respectively); lane 8: decatenated (dec) and linear (lin) DNA marker.

in the parental cell line at any of concentration used is consistently lower than that produced in the cell mutant *irs-2*. These findings are in line with the results obtained in the comet assay and show that ICRF-193 produces DNA damage per se in cultured mammalian cells.

Taken as a whole, these results might be interpreted as evidence that the binding of ICRF-193 to topo II might cause poisoning of the topoisomerase similar to that observed with typical topoisomerase inhibitors such as *m*-AMSA [3,5]. From this, we hypothesized that if ICRF-193 would act as a topo II poison, a reduction in DSB formation should be observed with the drug applied simultaneously together with an inhibitor of DNA synthesis, since the ability of topoisomerase poisons to produce DNA damage is related to their interaction with DNA replication fork progression [20]. To examine this possible mechanism of poisoning of the enzyme, PFGE measurements were carried out to detect DSB induced in cells treated during 3 h with ICRF-193, alone or in the presence of the DNA synthesis inhibitor aphidicolin (APH) to inhibit replication during treatment with the topo II inhibitor. *m*-AMSA, a well known topo II poison was used as positive control. As can be seen in Fig. 5A and B, the amount of DNA migrating from the well, here taken as a measure of DSB, was clearly higher when V79 or *irs-2* cells were treated with *m*-AMSA alone than when APH was present at the same time, in agreement with our previous observations with CHO cell lines [20]. However, in contrast to these results, no reduction in the amount of DNA released from the wells was observed after treatment with ICRF-193 when administered in the presence of APH. These results indicate that DSB are produced by ICRF-193 independently of the collision of DNA replication forks with the topo II enzyme–inhibitor complex.

4. Discussion

Until recently, it was believed that the genotoxic activities of topo II inhibitors depend on their capacity for stabilizing covalent cleavable complexes in DNA [3,21]. For example, complex-forming agents like amsancrine, doxorubicin, etoposide, etc. have been reported to be genotoxic, readily inducing chromosomal aberrations and sister-chromatid exchanges (SCEs) [22–26]. These inhibitors mainly accumulate cleavable DNA–topoisomerase complexes and the failure to resolve these before DNA replication results in the production of DSB, chromosomal aberrations and, finally, cell death. Indeed, several studies have established a firm relationship between the ability of cleavable complexes formation of these poisons and their cytotoxicity [27,28].

On the other hand, the mechanism(s) whereby the catalytic inhibitors of topoisomerases exert their cytotoxicity is far from being clear. Most studies of catalytic inhibitors have focused on identifying the catalytic step(s) at which they interact, as reviewed

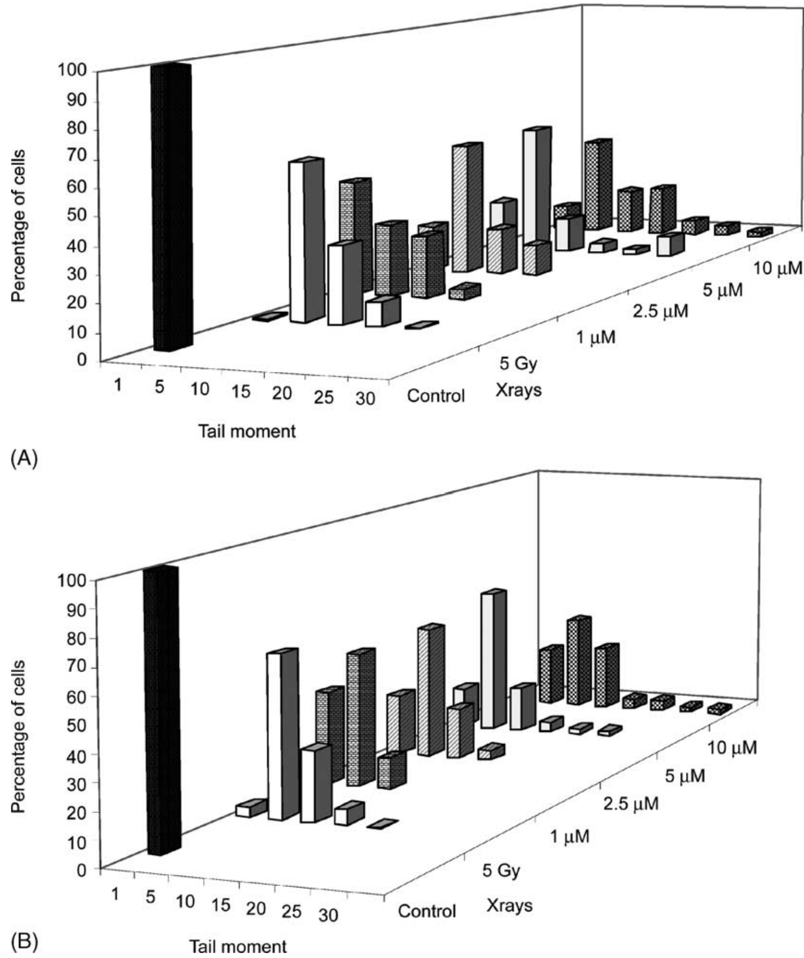

Fig. 3. Effectiveness of different concentrations of the topo II catalytic inhibitor ICRF-193 to induce DNA damage in V79 (A) and *irs-2* (B) cells, as shown by the Comet assay. Cells exposed to 5 Gy X-rays were used as a positive control. Data from three independent experiments (50 comets were measured per experimental point in each experiment). Observe the dose-dependent increase in tail moments, as compared to non-treated controls ($P < 0.0001$; Student's *t*-test).

recently by Andoh and Ishida [2], and only a few of such inhibitors have been investigated with the purpose of analyzing their cellular effects [29,30]. The bis-dioxopiperazine ICRF-193 is one of the topo II inhibitors considered as being purely catalytic based on results showing its ability to inhibit topo II activity without stabilization of the cleavable complex [9,30] as well as a reported inability to produce DNA strand breaks [31]. However, recently, evidence has been presented that this compound is acting as a novel type of topo II poison [10]. Huang et al. [11] have reported that ICRF-193 is able to produce DNA strand breaks using a chaotropic denaturing agent for damage detection. In this connection, it appeared of interest to characterize further the cellular effects of the novel topo II inhibitor ICRF-193 which is unable to stabilize cleavable complex formation but whose mechanism of action has been elucidated recently [4].

The observations presented here provide evidence that ICRF-193 exhibits DNA-damaging activity in both V79 cells and its radiosensitive cell mutant *irs-2*. The induction of DNA strand breaks was detected using two different techniques, namely single cell gel electrophoresis (comet assay) and pulsed-field

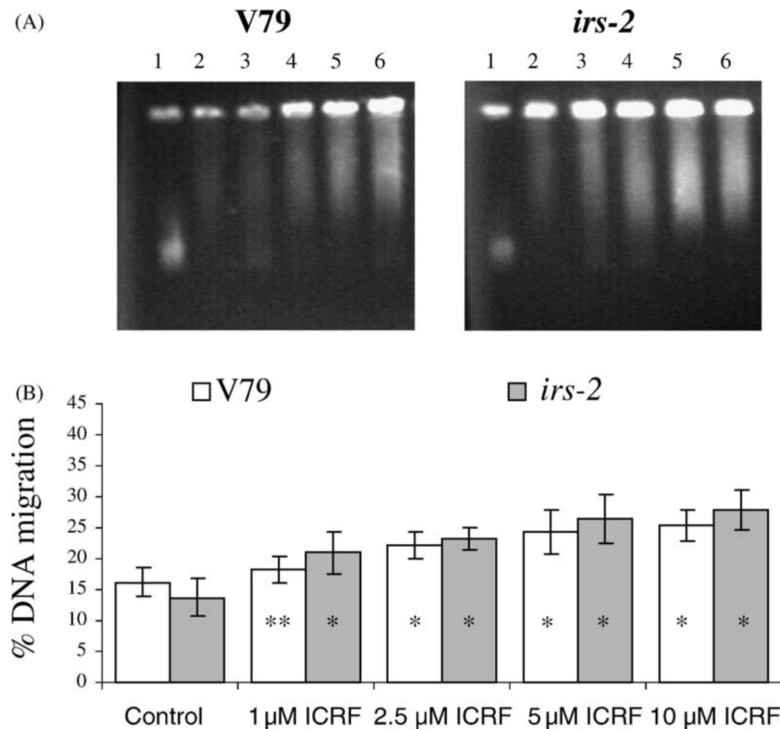

Fig. 4. Pulsed-field gel electrophoresis measurements of DNA double-strand breaks in V79 (□) and *irs-2* (■) cells exposed to 0, 1, 2.5, 5, or 10 μM ICRF-193. (A) Representative PFGE gels of three independent experiments are shown. Cells were included in 0.8% low-melting point agarose at a final density of 4×10^6 cells ml^{-1}. DNA from approximately 200,000 lysed cells was migrated by PFGE as described in Materials and Methods. Lane 1: *S. cerevisiae* DNA marker; lane 2: untreated controls; lanes 3–6: cells exposed to 1, 2.5, 5, and 10 μM ICRF-193. (B) PFGE analysis of DNA DSB produced by ICRF-193. Data are expressed as the percent of DNA migrated from the well which is proportional to the DNA DSB level. Each bar represents the mean of three independent experiments ± S.D. ($^{*}P < 0.001$, $^{**}P < 0.002$, according to Student's *t*-test).

gel electrophoresis. Moreover, ICRF-193 induced damage in a dose-dependent manner, and the extent of the damage produced in the mutant cell line was even somewhat higher at the concentrations tested. Furthermore, it is known that X-ray-sensitive cell lines are in general hypersensitive to DNA–topo II inhibitors such as etoposide, which stabilize topo II–DNA cleavable complexes and thus concomitantly induce double-strand breaks [5]. This hypersensitivity has generally been ascribed to the deficiency of these cell lines in DSB repair [6,7,32]. *irs-2* showed only moderate hypersensitivity to ICRF-193 in its capacity to generate DSB compared to the parental cell line. These data are in good agreement with those obtained earlier by Jones et al. [32] who used other topo II inhibitors. Unfortunately, to our knowledge no other data exist on the DSB induction by ICRF-193 in this radiosensitive cell line that could be compared to ours.

Taken as a whole, these results seem consistent with the cell viability data obtained in the SRB assay after 48 h of ICRF-193 treatment for both cell lines. Concerning this, the DNA damage induced by ICRF-193 in both cell lines observed by us might be associated with or result in the first events leading to the cytotoxicity of this topo II inhibitor.

On the other hand, the results obtained in the present paper challenges the previously published negative reports on the ability of catalytic inhibitors of topo II to induce DNA strand breaks. Notwithstanding, we would like to remark that these data have largely been based on results from in vitro assays using purified enzyme and isolated DNA such as supercoiled plasmid DNA or kinetoplast DNA [33,34] and up to date,

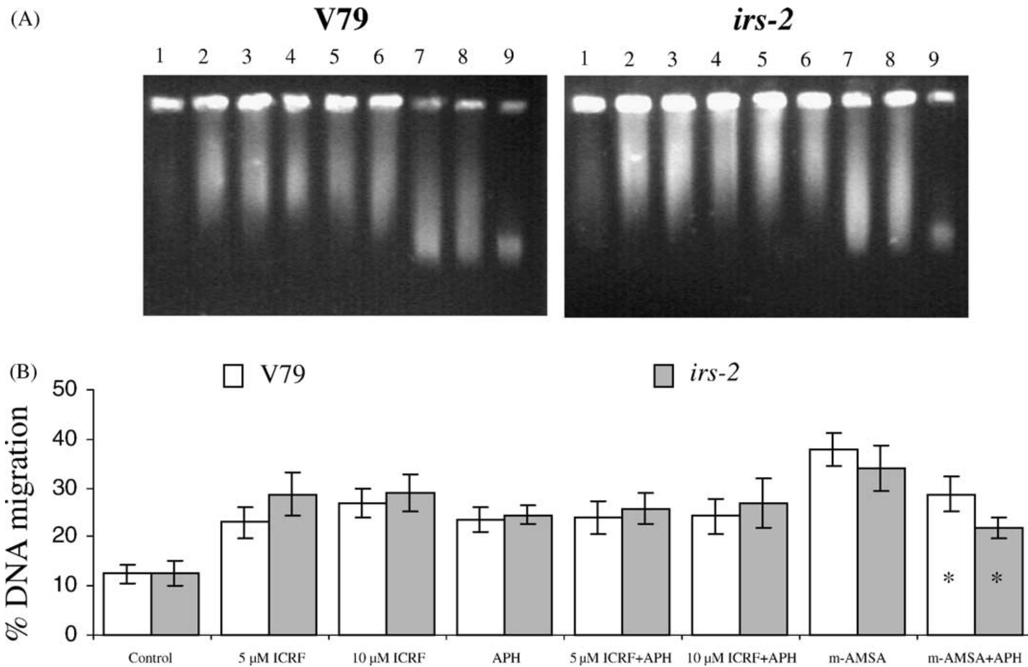

Fig. 5. Influence of the DNA synthesis inhibitor aphidicolin (APH) on DNA double-strand breaks produced by ICRF-193 or *m*-AMSA in V79 (□) and *irs-2* (■) cells. (A) Representative PFGE gels of three independent experiments are shown. Lane 1: untreated controls; lanes 2 and 3: cells exposed to 5 or 10 μM ICRF alone; lane 4: cells treated with 10 μM APH alone; lanes 5 and 6: cells exposed to 5 or 10 μM ICRF-193 and APH simultaneously; lane 7: cells exposed to 2 μM *m*-AMSA; lane 8: cells treated with 2 μM *m*-AMSA plus APH; lane 9: *S. cerevisiae* DNA marker. (B) PFGE analysis performed to quantify the effects of APH on DSB generated by ICRF-193 or *m*-AMSA. Data are expressed as the percent of DNA migrated from the well. Each bar represents the mean of three independent experiments ± S.D. Comparisons were realized between samples treated with APH + ICRF-193 or APH + *m*-AMSA and those treated with its respective topo II inhibitor alone. The statistical evaluation was done using Student's *t*-test. Note that the presence of APH does not influence DSB induced by ICRF-193 ($P > 0.2$). In contrast, a reduction in DSB generated by *m*-AMSA is observed when this topo II inhibitor is administered simultaneously with APH ($^*P < 0.001$).

very few data have been published using techniques as comet assay or PFGE to evaluate the capacity of topo II catalytic inhibitors to generate DNA damage in cells [35,36].

Focusing on ICRF-193, we have recently obtained data on the capacity of this topo II inhibitor to produce DNA strand breaks [37]. Furthermore, to our knowledge, only Boos and Stopper [35] have recently reported a weak DNA strand break ability of this topo II inhibitor also using the comet assay for DNA damage evaluation. On the other hand, negative results using PFGE had been reported earlier by Muñoz et al. [38]. The basis for the discrepancy between our strongly positive data and the negative reports is not clear, but may be the result of differences in treatment conditions or technique sensitivity. Woudstra et al. [39] have examined the damage levels detected in two cell lines by a range of methods, and the results suggest that different assays can lead to different conclusions. In this respect, it is known that the ability of the comet assay to detect certain types of DNA lesions is influenced by assay conditions. Thus, the negative results previously reported could indicate that the conditions used were not sensitive enough at detecting DNA damage. Olive [40] has recently suggested that even identical lesions may be recognized differently by different cells, which may add even a further level of complexity. Therefore, heterogeneity in the type of DNA damage may also be an important problem.

Surprisingly, the picture changes when the clastogenicity of ICRF-193 has been analyzed. Thus, it is remarkable that Boss and Stopper [35] only observed

a weak induction of DNA damage by ICRF-193 in the comet assay whereas it was an effective inducer of micronuclei. In good agreement with these results we have recently reported evidence that shows the ability of ICRF-193 in breaking both the DNA and the chromosomes of Chinese hamster AA8 and EM9 cells [37]. Earlier, Ikushima et al. [41] had also reported on the effectiveness of the topo II catalytic inhibitor ICRF-193 to induce chromosome and chromatid-type aberrations with high frequencies in Chinese hamster V79 cells. Furthermore, it has recently been demonstrated that the inhibition of topoisomerase II activity by ICRF-193 during meiosis II was responsible for induction of structural chromosome aberrations and aneuploidy in mouse oocytes [42]. From the results stated above, it appears that the clastogenic activity of ICRF-193 correlates well with our results obtained on the induction of DNA strand breaks.

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 [9] but it catalytically inhibits mammalian DNA–topo II in a rather unique manner. 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 [4].

There is a considerable controversy with regard to the mechanism(s) by which ICRF-193 might kill cells. Although our results indicate that the enzyme is able to cleave DNA and therefore, could be considered to act as a poison, in our opinion it does not seems likely that ICRF-193 acts as a complex-stabilizing topoisomerase II poison. In favor of this are the results obtained by PFGE analysis on the absence of DNA breakage reduction in V79 and *irs-2* cells after a treatment with ICRF-193 when using an inhibitor of DNA synthesis (APH). On the contrary, a reduction in DNA migration was observed when *m*-AMSA, a topo II poison, was employed in the combined treatment compared to those cells which received only the treatment with *m*-AMSA [20]. Therefore, this experiment demonstrates that the deleterious effect(s) on genomic DNA of ICRF-193 was clearly produced in a manner independent of the collision between DNA replication forks and stabilized topo II enzyme–inhibitor complex. A plausible explanation according to the evidences reported here should be that the stabilized closed clamp-form of the enzyme might impede DNA metabolic events, 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 [10].

In conclusion, although the mechanism(s) responsible for the genotoxicity and cytotoxicity of ICRF-193 remains to be fully elucidated, the results from the present work support that this topo II inhibitor exerts DNA-damaging activity in mammalian cells [35,37,41,42]. Although the particular nature of this damage is unknown, our data suggest that it could be involved, at least in part, in mediating the cytotoxicity of this topo II inhibitor. It is worth mentioning that, for the first time, a replication-independent production of DSB by ICRF-193 as assessed by PFGE is herein reported. In our opinion, these latter features deserve special attention and constitute the major novelty of the present investigation.

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