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Mutation Research 599 (2006) 105–115
Fundamental and Molecular Mechanisms of Mutagenesis
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Modulation of radiation response by inhibiting topoisomerase II catalytic activity

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Received 28 July 2005; received in revised form 9 February 2006; accepted 14 February 2006
Available online 30 March 2006

Abstract

Due to the essential role played by DNA topoisomerases (topos) in cell survival, the use of topoisomerase inhibitors as chemotherapeutic drugs in combination with radiation has become a common strategy for the treatment of cancer. Catalytic inhibitors of these enzymes would be promising to improve the effectiveness of radiation and therefore, it appears reasonable to incorporate them in combined modality trials. In this work, we have investigated the capacity of both ICRF-193 and Aclarubicin (ACLA), two catalytic inhibitors of topoisomerase II (Topo II), to modulate radiation response in Chinese hamster V79 cell line and its radiosensitive mutant *irs2*. We also have explored potential mechanisms underlying these interactions. Experiments were performed in the presence and absence of either ICRF-193 or ACLA, and topo II activity was measured using an assay based upon decatenation of kinetoplast DNA (kDNA). For the combined experiments cells were incubated for 3 h in the presence of various inhibitor concentrations and irradiated 30 min prior to the end of treatments and cell survival was determined by clonogenic assay. DNA-damaging activity was measured by single-cell gel electrophoresis. Our results demonstrate that combinations of catalytic inhibitors of topo II and radiation produce an increase in cell killing induced by ionising radiation. The mechanism of radiation enhancement may involve a direct or indirect participation of topo II in the repair of radiation-induced DNA damage.
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Keywords: Radiation; Topoisomerase II inhibitors; ICRF-193; Aclarubicin; Cytotoxicity; DNA damage

1. Introduction

Radiation therapy has traditionally been the treatment of choice for locally or regionally advanced surgically unresectable cancers. Even though technologic and methodologic improvements have been frequent, the rate of local failures remains high, and overall patient survival is low. To improve therapeutic results, radiation is often combined with chemotherapy and a number of strategies are under intensive investigation, including searching for drugs that are more cytotoxic to tumour cells on their own or more effective as radiosensitizers, avoiding or selectively preventing normal tissue injury by radiation or drugs [1–3].

Due to the essential role played by DNA topoisomerases (topos) in cell survival, the use of topoisomerase poisons as chemotherapeutic drugs in combination with radiation has become a common strategy for the treatment of cancer. A number of clinically important agents capable of inhibiting the topo I and II are amongst the more effective antineoplasic drugs used in

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doi:10.1016/j.mrfmmm.2006.02.002

cancer therapy. The clinical use of a number of topoisomerase poisons such as camptothecin, topotecan, or beta-lapachone that potentiate the cytotoxicity of agents that produce DNA damage as methyl-methanesulfonate (MMS) [4], neocarcinostatin [5], and X-rays [6,7] is well documented. These drugs kill cells by poisoning the topoisomerase catalytic cycle. In fact, they stabilize the transient covalent topoisomerase-cleaved DNA intermediates, which seem to trigger a chain of events causing cell death.

Another class referred to as catalytic inhibitors, which characteristically do not stabilize but can antagonize the formation of cleavable complexes comprises aclarubicin (ACLA), the bis(dioxopiperazines), fostriecin, suramin and merbarone, among other drugs [8]. These catalytic inhibitors express a variety of inhibitory mechanisms. For example, ACLA intercalates DNA and is thought to prevent its binding to the topoisomerase enzyme [9], while ICRF-193 has been shown to trap yeast topo II in a closed clamp conformation [10], thereby acting at the postreligation step of the catalytic cycle. One noticeable difference between topo II poisons and catalytic inhibitors is that while the former have supplied some of the most clinically potent and widely used anticancer drugs, the only catalytic inhibitor that has been successful so far in clinical oncological practice, mainly in the treatment of leukemia, has been the intercalative drug ACLA [11].

The goal of the present study was to investigate the ability of a treatment with ACLA, a dual inhibitor of both topoisomerases I and II and ICRF-193, a topo II inhibitor, to enhance the lethal effects of ionising radiation. The radiosentitive Chinese hamster cell line *irs2*, phenotypically similar to the human cancer-prone syndrome ataxia telangiectasia (A-T) [12], displays a highly sensitivity to the topo I poison camptothecin [13,14], while in constrast it shows little or no increased sensitivity to topo II inhibitors [14]. In the present investigation we further wanted to assess the possible different behaviour of parental V79 and *irs2* cells concerning their response to these two catalytic inhibitors which, in turn, would help us to delineate the mechanistic basis for their cytotoxic activities.

2. Materials and methods

2.1. Cell culture

The parental lung fibroblast Chinese hamster cell line V79 was purchased from the American Type Culture Collection (ATCC), USA. The radiosensitive 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) and streptomycin (50 μg/ml). Cells were cultured at 37 °C in an atmosphere containing 5% CO2. On regular testing, cell cultures were found to be free from mycoplasma.

2.2. Irradiation and colony formation

Cell survival following ionising irradiation was measured by clonogenic assay in monolayer. Cells were harvested and suspended in full culture medium. Single-cell suspensions were plated out at appropriate densities in triplicate. In all the experiments, cells in exponential growth were irradiated using an X-ray irradiator (Philips MU 15F) operated at 100 KV and a dose rate of 1 Gy/min. For the dosimetric study of V79 and *irs2*, irradiations were performed 4 h after plating, when cells were attached. For the combined experiments (topo inhibitor + X-rays) cells were incubated for 3 h in the presence of different inhibitor concentrations and irradiated 30 min prior to the end of treatments. The concentration range tested was from 0.1 to 1 μg/ml for ACLA and from 1 to 10 μM in the case of ICRF-193, respectively. Control dishes were seeded at 200 cells/dish, while those treated were plated at higher cell densities to cope with the increased reproductive death. In all the experiments, cells were incubated in complete culture medium at 37 °C for 7–10 days after irradiation. Eventually, the medium was aspirated and dishes were fixed in methanol and stained with 3% Giemsa for 30 min, then rinsed and air-dried. Surviving colonies made up of more than 50 cells per colony were counted. Survival data are shown for correction for untreated cell cloning efficiencies. Data were fitted on a semi-log plot and three separate experiments were carried out for each cell line.

2.3. Preparation of nuclear extracts

Exponentially growing V79 and *irs2* cells were incubated for 3 h in the presence of different concentrations of ICRF-193 (0.5, 1, 2.5, 5, and 10 μM) or ACLA (0.05, 0.1 1 and 2 μg/ml), respectively. 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 lysed by adding 100 μl of 2 M NaCl, 20 mM Tris–HCl pH 7.5, 10 mM β-mercaptoethanol, 1 mM PMSF. Following 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.

2.4. 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). A 100 ng 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 or aclarubicin (0.05, 0.1, 1 and 2 μg/ml), respectively. 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.

2.5. Comet assay

V79 and *irs2* were treated for 3 h with selected concentrations (1, 5 and 10 μM) of ICRF-193 or aclarubicin (1 and 2 μg/ml), respectively. Just before the end of the treatments, untreated and treated cells were irradiated on ice with a dose of 5 Gy of X-rays. Following ionising irradiation, DNA damage was measured by the alkaline single-cell gel electrophoresis or comet assay.

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 lysing 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 lysing 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 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) 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 the image analysis CASys software (Synoptics Ltd., image processing systems, UK) [19]. The measure of damage was the 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.

2.6. Statistical analysis

From the experimental results, a mean value and standard error of the means (S.E.M.) were calculated for each experimental group. Student's *t* test with the resultant *p* value representing a two-sided test of statistical significance was used. Significance was set at 95% ($p = 0.05$).

3. Results

3.1. Cell survival

The clonogenic cell survival response for the parental cell line V79 and its cell mutant *irs2* to different doses of ionising radiation are shown in Fig. 1. The linear-quadratic equation $\ln \mathrm{SF} = -(\alpha D + \beta D^2)$ was fitted to the data using non-linear regression analysis. It is apparent that there are marked differences in radiosensitivity between both cell lines, with the cell mutant being more sensitive than the parental cell line. Also it can be seen a fifty percent reduction in cell survival when the mutant *irs2* was exposed to a dose of 1 Gy of X-rays. However, it was necessary a dose of ionising radiation five times higher in order to obtain a similar decrease in cell survival for the parental V79 cell line. Accordingly, these doses of X-rays (1 Gy for *irs2* and 5 Gy for V79) which reduced cell survival to similar values in both cell lines

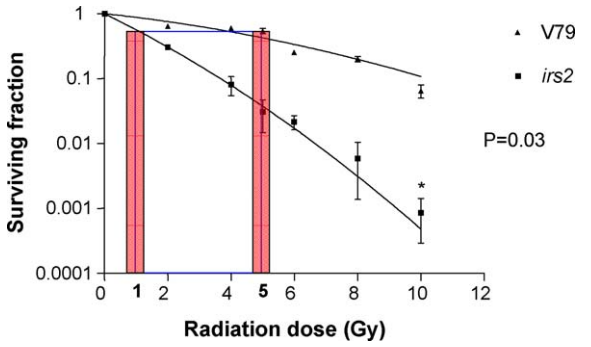

Fig. 1. Survival curves of V79 (▲) and *irs2* (■) cells. The linear-quadratic equation $\ln SF = -(\alpha D + \beta D^2)$ was fitted to the data using non-linear regression analysis. Single-cell suspensions were plated out at appropiate densities before exposure to increasing doses of X-rays (0–10 Gy). Differences in radiosensitivity can clearly be observed between both cell lines ($p = 0.03$), with the cell mutant being more sensitive than the parental cell line. Doses of X-rays (1 Gy to *irs2* and 5 Gy to V79; red bar) which reduced cell survival to similar values in both cell lines were those chosen for combined experiments. The data represents the mean ± S.E.M. from three independent experiments (for interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

were those chosen when we wanted to compare the lethal effects produced by X-rays under conditions of topo II inhibition (combined treatment topo II inhibitor + X-rays).

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

The enzyme capacity to decatenate double-stranded catenated kinetoplast DNA (kDNA) was the endpoint used to assess the inhibition of topo II catalytic activity by ICRF-193 (Fig. 2) or the intercalative anthracycline ACLA (Fig. 3). As can be seen, in the absence of any drug treatment, topo II activity recovered in nuclear extracts from both V79 and *irs2* was able to efficiently decatenate the catenated DNA substrate made up of interlocking rings of double-stranded DNA, as shown by the release of closed minicircles. Also, Figs. 2 and 3 show that increasing concentrations of ICRF-193 or ACLA produce an inhibition of topo II catalytic activity in both cell lines, as shown by a progressive increase in the amount of catenated DNA substrate remaining in the wells. The image densitometry showed no significant differences between the repair-proficient parental V79 cells and the radiosensitive *irs2* mutant with respect to the inhibitory effect of ICRF-193 (Fig. 2) or ACLA (Fig. 3) on topo II catalytic activity in the dose range tested. The doses assayed comprised from 0.5 to 5 μM for ICRF-193 or from 0.05 to 2 μg/ml for ACLA. On the basis of these results, dose intervals were selected for the experiments designed to investigate the capacity of ICRF-193 or ACLA to enhance the cell death produced by X-rays in both cell lines.

3.3. Effects of DNA topoisomerase inhibitors ICRF-193 or ACLA on X-ray cell survival

The ability of a pre-treatment with ICRF-193 (1–10 μM) or ACLA (0.1–0.5 μg/ml) to potentiate the lethal effect induced by X-rays in V79 and *irs2* cells is shown in Fig. 4. On the other hand, the effect of different concentrations of ICRF-193 (0.1–25 μM) or ACLA (0.1–1 μg/ml) on cell survival is show in Fig. 4A and B, respectively. Data show a small but consistently higher cytotoxic effect produced by the treatment with topoisomerase inhibitors for the radiosensitive cell mutant *irs2* compared to the parental V79 cell line ($p < 0.05$, Student's *t*-test).

Comparisons were made between the clonogenic capacity of cells irradiated with 5 Gy (V79) or 1 Gy (*irs2*) of X-rays (under conditions of normal topo II catalytic activity) and those that had been pre-treated for 3 h with different concentrations of ICRF-193 (Fig. 4C) or ACLA (Fig. 4D) so, irradiated under conditions of partial inhibition of topo II catalytic activity. Results show for both cell lines a clear decrease in cell survival values for those cells irradiated when the topo II catalytic activity had been depleted to some degree as compared to the survival cell values for control irradiated cells. Cell survival data for both cell lines were significantly different from their respective controls according to Student's *t*-test comparison ($p < 0.01$). In addition, it can be concluded that for each of the ACLA concentrations used, the loss in its capacity to form colonies was significantly higher ($p < 0.05$) for the mutant *irs2* compared to the parental V79 cell line only when cells were pre-treated with the topoisomerase inhibitor ACLA (Fig. 4D). However, no statistical differences were observed between cell lines when cells were pre-treated with ICRF-193 (Fig. 4C).

3.4. Effects of DNA topoisomerase inhibitors ICRF-193 or ACLA on DNA damage induced by X-ray

The effectiveness of a pre-treatment with ICRF-193 (1, 5 and 10 μM) or ACLA (1 and 2 μg/ml) to add to the DNA damage induced by 5 Gy of X-rays in V79 and *irs2* cells is shown in Fig. 5A and B, respectively. The inhibitor concentrations were selected on the basis of its ability to inhibit the topo II catalytic activity but

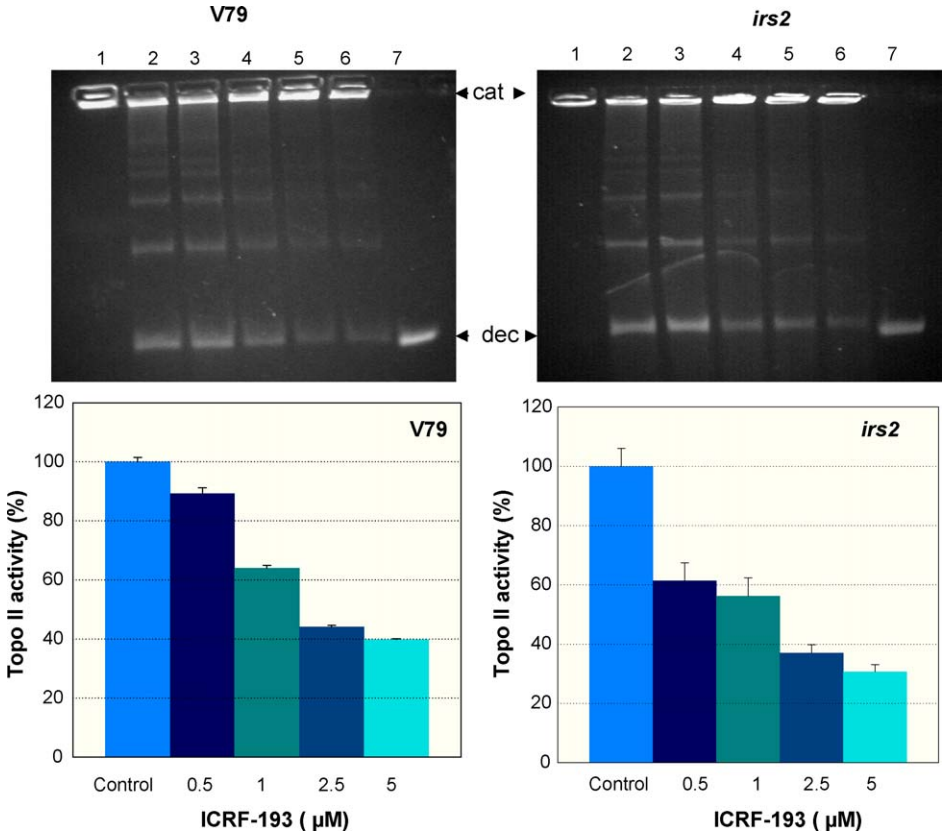

Fig. 2. Efectiveness of different doses of ICRF-193, ranging from 0.5 to 5 μM to inhibit topo II catalytic activity. Nuclear extracts from V79 and *irs2* cells were obtained as described in Section 2 and their ability to decatenate catenated kinetoplast DNA was assayed by DNA gel electrophoresis. Lane 1: marker catenated (cat) kinetoplast DNA; lane 2: control non-treated with ICRF-193; lanes 3–6: treated with increasing concentrations of ICRF-193 (0.5, 1, 2.5 and 5 μM, respectively); lane 7: decatenated (dec) DNA marker. The intermediate bands in the gels represent partial (incomplete) digestion of the catenate DNA substrate. Densitometric profiles for both cell lines are shown below. The loss of topo II catalytic activity in ICRF-193-treated cells was in all the cases significant compared with non-treated cells ($p < 0.01$; Student's *t*-test).

taking care that they could allow in all cases reasonable levels of cell viability. Calculation of the potentation factor (PF) was done according to the formula: $PF = (E_{X\text{-rays+inhibitor}} - E_{inhibitor})/(E_{X\text{-rays}} - E_c)$, where $E_{X\text{-rays}}$ is the effect of X-rays alone, $E_{X\text{-rays+inhibitor}}$ the effect of the combined treatment, $E_{inhibitor}$ the effect of the inhibitor (ICRF-193 or ACLA) alone, and E_c the DNA damage in the untreated control. A potentation factor of 1 corresponds to no potentiation. Any PF value from 1 to 1.5 corresponds to an additive effect while PF > 1.5 corresponds to a synergistic effect. Our results represented in Fig. 5A and B show for all the combinations analysed that only a slight synergism was found for the combination of 1 μM ICRF-193 with X-rays (FP = 1.56), whilst additivity was found for the rest of combinations assayed. Overall, these data indicate the general pattern of these combinations as additive, showing a similar behaviour in both cell types.

4. Discussion

Combined chemoradiation has been demonstrated to improve treatment outcome as compared to radiation alone in a number of different malignancies [20–23]. However, the therapeutic improvements credited to these regimens have been achieved using standard chemotherapeutic agents, such as cisplatin and 5-FU, which have traditionally been selected for combined treatment based primarily on their known clinical activity in particular disease sites. Unfortunately, chemoradiotherapy using these agents is limited in its application, because it is usually associated with increased normal tissue toxicity. Therefore, there is an urgent need to develop strategies to enhance the efficacy of radiotherapy. In this respect, the use of classical topoisomerase poisons in combination with radiation or chemicals has been considered promising in cancer therapy. Different studies have shown how several topoisomerase poisons such as camptothecin,

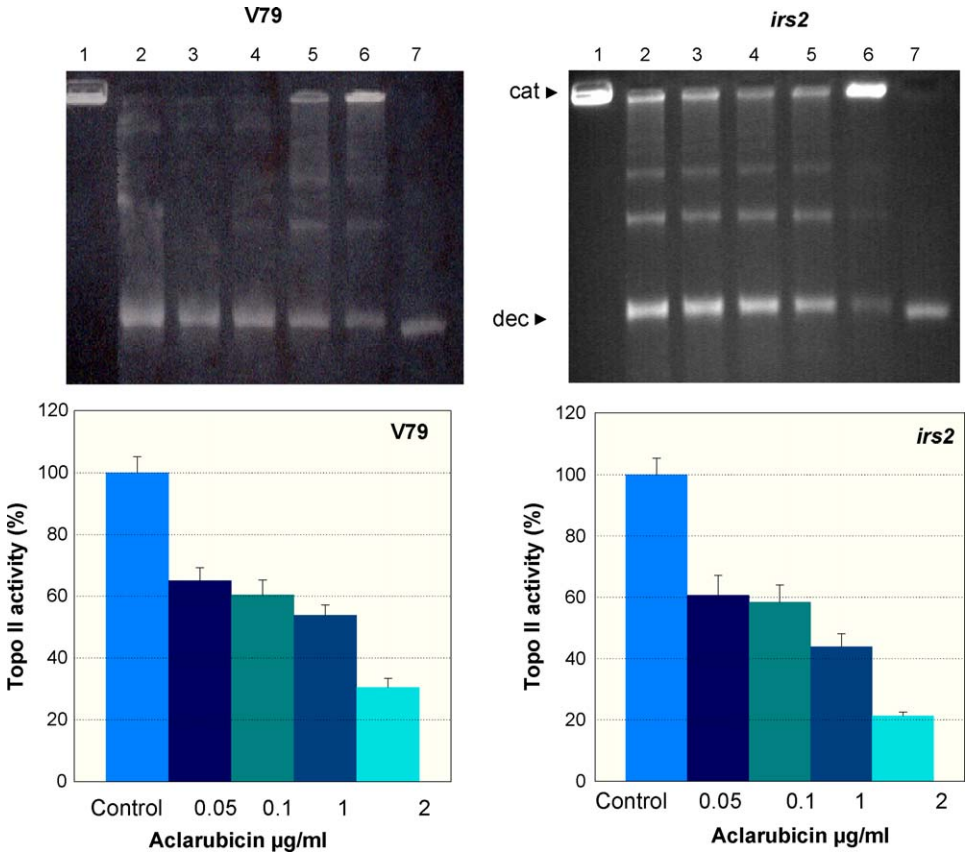

Fig. 3. Efectiveness of different doses of ACLA, ranging from 0.05 to 2 μg/ml to inhibit the topo II activity. Nuclear extracts from V79 and *irs2* were obtained as described in Section 2 and their ability to decatenate catenated kinetoplast DNA was assayed by DNA gel electrophoresis. Lane 1: marker catenated (cat) kinetoplast DNA; lane 2: control cells not treated with ACLA; lanes 3–6 treated with increasing concentrations of ACLA (0.05, 0.1, 1 and 2 μg/ml, respectively); lane 7: decatenated (dec) DNA marker. The intermediate bands in the gels represent partial (incomplete) digestion of the catenate DNA substrate. Below, the respective densitometric profiles are shown. The loss of topo II catalytic activity in ACLA-treated cells was in all the cases significant compared with non-treated cells ($p < 0.01$; Student's *t*-test).

beta-lapachone or topotecan, are able to potentiate the cytotoxicity produced by methyl-methane-sulfonate [4] neocarcinostatin [5] or X-rays [6,7,24].

Recently, catalytic inhibitors of DNA topo II are being studied as a novel class of anticancer agents but, up to now, research has been scarce. It is worth mentioning the work from Barret's laboratory using F11782, a new dual topo I poison and topo II catalytic inhibitor [25–27]. Improved results were obtained using different combinations of F11782 plus several known anticancer drugs such as etoposide, cisplatin, mitomycin C or doxorubicin [25–27].

Following this lead, in the present paper we have studied the ability of two catalytic inhibitors of topo II to enhance the toxicity produced by X-rays. Our results illustrated in Fig. 4 show that the combination of ACLA or ICRF-193 plus X-rays leads to a dose-dependent increase of the cytotoxicity induced by ionising radiation in both V79 and the radiosensitive cell line *irs2*. The inhibitors concentrations were selected on the basis of their ability to inhibit to a higher or lesser extent the topo II activity (Figs. 2 and 3). Data from Fig. 4 also show a decrease in cell survival values for all combinations assayed, being these values significantly lower for the *irs2* cell mutant as compared to the parental cell line V79. On the other hand, our results concerning any possible differences between the radiosensitive mutant *irs2* and its parental line V79 in either the basal topo II (Figs. 2 and 3) or their response to ICRF-193 or ACLA (Fig. 4A and B), respectively in terms of enzyme inhibition, show a rather similar picture for both cell lines. This observation, on the other hand, is in good agreement with the lack of differences in topo I activity between V79 and *irs2* reported by Jones et al. [14], and seems to support the idea that whatever the differences between both cell lines in their sensitivity to topo I or topo II inhibitors,

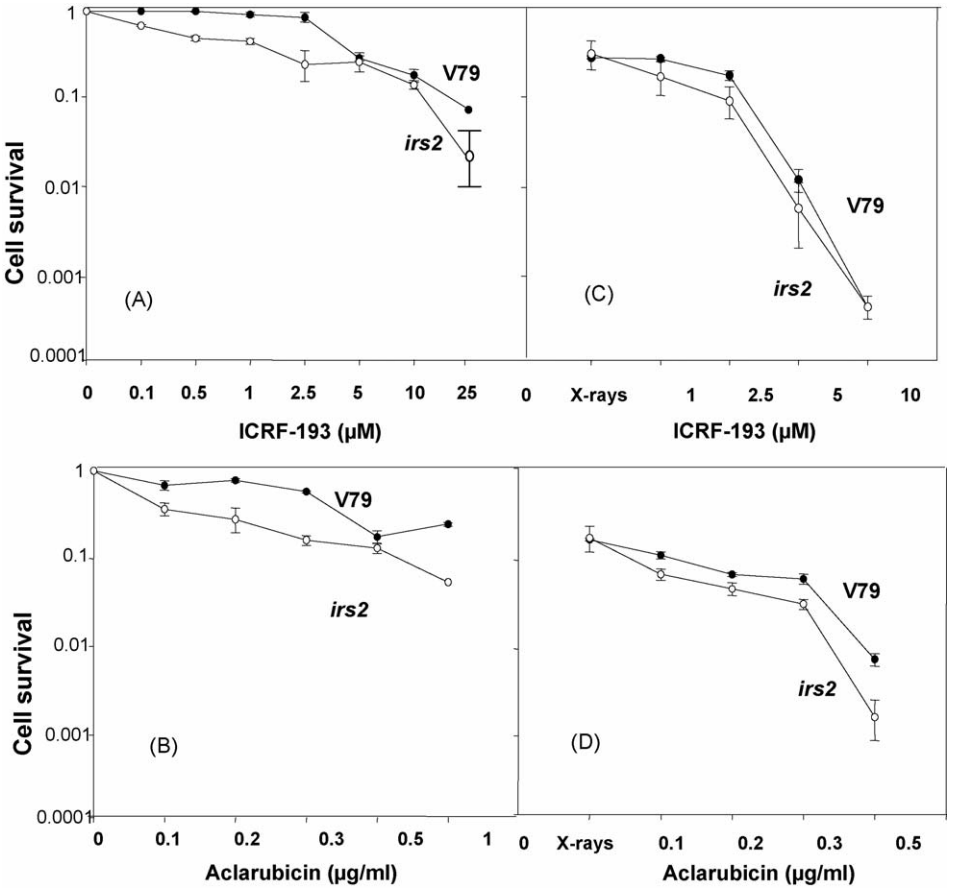

Fig. 4. Efectiveness of topoisomerase II inhibitors to modulate radiation response. The influence of different concentrations of ICRF-193 or ACLA on cell survival is depicted in (A) and (B), respectively. V79 and *irs2* cells were treated with increasing concentrations of ICRF-193 or ACLA for 24 h and then grown for 7–10 days in normal media. Data show a higher sensitivity to both topo II inhibitors for the radiosensitive mutant *irs2* as compared to the parental V79 cell line ($p < 0.05$; Student's *t*-test). The possible effectiveness of combining ICRF-193 (C) or ACLA (D) and radiation was examined by clonogenic survival in V79 and *irs2* cells after various doses of catalytic inhibitors as described in Section 2. Control cells exposed to X-rays (5 Gy to V79 or 1 Gy to *irs2*) without any previous topo II inhibitor treatment induced a similar decay in cell survival values. However, combined treatment with topo II inhibitors and radiation resulted in a significant reduction in clonogenic capacity of both V79 and *irs2* cells compared to control cells ($p < 0.01$; Student's *t*-test). Data are given as the mean ± S.E.M. from three independent experiments.

cannot be ascribed to any enzyme abnormality. Besides, these results are partly in agreement with those reported above from Barret's group and add new data about the possibility to enhance the mitotic cell death induced by ionising radiation through a modulation of the topo II activity of the cell. However, it is worth mentioning that the increase observed by us for the combined experiments was in general additive, contrasting with the strong synergistic effect showed when F 11782, a novel dual inhibitor of topo I and II was combined with diferent anticancer agents [27].

In our opinion, the data presented here suggest that a combination of X-rays and topo II inhibitors (at least those tested in the present study) is unlikely to provide improved regimes for clinical treatment of tumours. In this sense, it has been also shown that, depending on the cell line used, potentiating or antagonistic effects can be found for the combinations of topoisomerase inhibitors such as etoposide, topotecan or SN38 plus paclitaxel (PTX) [28–32]. Overall, these results suggest the necessary caution that must be taken before doing generalizations about the different combinations of topoisomerase inhibitors and DNA damaging agents, taking into account the different actions they may have in different cell lines.

At the molecular level, ionising radiation induces a vast number of damage types in DNA. Small-base or nucleotide damage lie at one side of the spectrum with single- and double-strand breaks, while multiply damaged sites lies at the other. DNA double-strand breaks

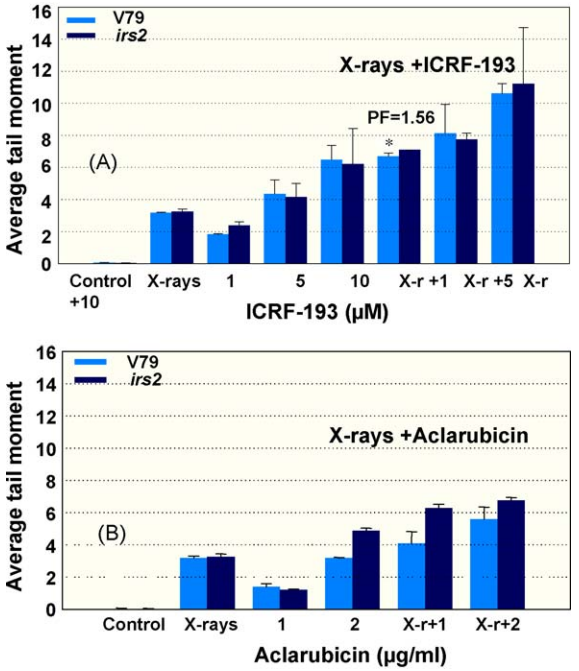

Fig. 5. Effectiveness of different combinations of ICRF-193 (A) or ACLA (B) to modulate the DNA damage induced by radiation in V79 and *irs2* cells, as shown by the comet assay. Pre-treatment with topo II inhibitors resulted in a synergistic enhancement of radiation DNA damage (potentiation factor PF = 1.56; see Section 3 for PF calculations) only for the combination of 1 μM ICRF-193 and radiation, whilst additivity was found for the rest of combinations assayed. Data from three independent experiments are presented (50 comets were measured per experimental point in each experiment).

(DSBs) have been considered to be the most important type of lesion for the cytotoxic effects of radiation based on findings suggesting that their levels vary in directions consistent with killing [33,34]. Concerning the mechanism of cytotoxic interaction between drugs and ionising radiation, due to the heterogeneity in the type of DNA damage generated by radiation and the complexity of the possible chemical interactions between radiation and drugs, understanding the mechanism of cytotoxic interaction is very intrincate since so many factors can contribute to enhance radiocytotoxicity. For example, by increasing the initial damage, by affecting the capacity to repair DNA damage, altering the cell cycle or even affecting the availability of repair machinery at target sites.

The bis-dioxopiperazine ICRF-193 acts on topo II activity without formation of any cleavable complex [35] 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 complex, after passage of the intact DNA duplex, by inhibiting the intrinsic ATPase activity of the topo II, sequestering the enzyme from its normal turnover inside the cell [10]. In a previous paper we have demonstrated the DNA-damaging activity of this compound by a mechanism independent of the cell replication machinery, which partly would be responsible of its cytotoxic action [36]. On the other hand, ACLA is a DNA binding anthracycline antibiotic that inhibits the catalytic activity of topo II and simultaneously acts as a topo I poison able to stabilize topo I-DNA cleavable complexes with the subsequent production of DNA strand breaks [8,37–39]. Its anticancer performance has been ascribed to this latter property of the anthracycline, in a similar fashion to that reported for camptothecin [40–42]. Previous work demonstrated that camptothecin derivatives radiosensitized human cancer cells, and this effect was induced when drug treatment was given prior, but not following radiation [43–45].

Furthermore, recently it has been reported that the combination of RFS-2000 or CPT-11 plus etoposide is more effective than either agent separately in the enhancement of radiation effects in human carcinoma cells [46].

The significant enhancement of cell killing produced by the combined treatment ICRF-193 or ACLA plus X-rays would be difficult to ascribe to an increase in the initial DNA damage produced by X-rays under conditions of a depleted topo II catalytic activity, since results presented in Fig. 5 on the quantification of DNA damage assayed by the comet assay show an absence of potentation of the levels of DNA damage induced by X-rays when the topo II catalytic activity was partly inhibited with either ICRF-193 or ACLA, respectively. In this respect, the timing sequence of combined radiation and drug treatment is important for a proper radiation enhancement. The radiation enhancing effect was observed when drug treatment was given prior to radiation. Based on this data, a plausible explanation for the decrease in cell survival observed for the combined treatments would be the result of restarting cell replication processes under conditions of topo II catalytic activity depletion as a result of treatment with ICRF-193 (Fig. 2) or ACLA (Fig. 3) after the arrest of DNA replication produced by ionising radiation. This event probably would lead to the production of un-repaired lesions most likely due to the impossibility to count with an enough number of active enzyme molecules able to resolve the topological problems arisen during DNA replication fork progression.

In favour of this hypothesis are our data (Fig. 4C and D) concerning the higher effectiveness of both ICRF-193 and ACLA to enhance radiation killing in the cell mutant

irs2, which seems consistent with its radiosensitive phenotype. This cell mutant belongs to the *XRCC8* group showing similarities with cultured cells from the human cancer-prone syndrome ataxia telangiectasia (AT) [12]. Chinese hamster *XRCC8* mutants and AT cell lines display hypersensitivity to ionising radiation and camptothecin, have no apparent inability to rejoin single- or double-strands breaks, and display normal V(D)J recombination [13,14,47,48]. Interestingly, like AT cells hamster *irs2* cells display radioresistant DNA synthesis [13,48], this later characteristic allowing *irs2* cells to skip the normal arrest of DNA replication after radiation treatment. It is well known that radiation typically produces an arrest in G2. Among the various events that occur in the G2-phase, there is also an intensive process of DNA repair involving DNA synthesis [49–51] and this arrest allows the repair of DNA damage (cell cycle checkpoints). Therefore, the radioresistant DNA synthesis displayed by the *irs2* cell mutant probably would be the responsible for the higher levels of cell death found as compared to the parental V79 cell line. A possible explanation is that this might be a consequence of a higher accumulation of un-repaired DNA lesions due to a shortening of the time available for repair.

On the other hand, which role, if any, might topo II play in the recovery from radiation damage [52,53] is not yet known. One might speculate that topo II, which is associated with, or forms part of the nuclear matrix and may be located at the base of chromatin loop domains [54], alters DNA topology to make chromatin accessible to repair enzymes. ACLA and ICRF-193, by causing a depletion of the number of active catalytic molecules of enzyme might disturb a possible role for topo II in the repair of DNA-strand breaks induced by ionising radiation, so increasing cell killing. This could occur through an interference with a direct participation of this enzyme in DNA repair processes or, alternatively, the binding of repair complexes might be influenced to some extent by the presence of topo II in a cooperative fashion. According to these considerations, evidence in favour of a possible involvement of topo II in radiation damage repair comes from a recent work carried out in our laboratory in which we observed that X-irradiated CHO cells pre-treated with the topo II suppressor bufalin, did show a dramatic delay in their DNA repair kinetics, as assessed by the comet assay [55].

The current data demonstrate that combinations of catalytic inhibitors of topo II (ICRF-193 or ACLA) and radiation are effective to enhance the cell killing induced by ionising radiation alone. The mechanism of radiation enhancement may involve a direct or indirect participation of topo II in the repair of radiation-induced DNA damage.

Acknowledgements

This work has been partly funded by Grants from Spanish Ministry of Education and Science (BFU2004-0084), and Junta de Andalucía (CVI 120), Spain.

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