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Induction of genotoxic and cytotoxic damage by aclarubicin, a dual topoisomerase inhibitor

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Abstract

The anthracycline aclarubicin (ACLA) is an intercalative antibiotic and antineoplastic agent that efficiently binds to DNA, leading to a secondary inhibition of the catalytic activity of topoisomerase II (topo II) on DNA. Besides this activity, ACLA has been reported to exert a concomitant poisoning effect on topo I, in a fashion similar to that of the antitumor drug camptothecin and its derivatives. As a consequence of this dual (topo II catalytic inhibiting/topo I poisoning) activity of ACLA, the picture is somewhat confusing with regards to DNA damage and cytotoxicity. We studied the capacity of ACLA to induce catalytic inhibition of topo II as well as cytotoxic effects and DNA damage in cultured Chinese hamster V79 cells and their radiosensitive counterparts *irs-2*. The ultimate purpose was to find out whether differences could be observed between the two cell lines in their response to ACLA, as has been widely reported for radiosensitive cells treated with topo poisons. Our results seem to agree with the view that the radiosensitive *irs-2* cells appear as hypersensitive ACLA as compared with radiation repair-proficient V79 cells. The recovery after ACLA treatment was also followed-up, and the *irs-2* mutant was found to be less proficient than V79 to repair DNA strand breaks induced by ACLA.

Keywords: Aclarubicin; Topoisomerase II; Topoisomerase I; Cytotoxicity; DNA damage

1. Introduction

Topoisomerase II (topo II)-targeting drugs are classified into either topo II poisons, which play a role in stabilizing the otherwise fleeting enzyme-DNA intermediates, the so-called 'cleavable complexes', or topo II catalytic inhibitors. While topo II poisons are well known for their ability to efficiently induce DNA strand breaks [1], catalytic inhibitors generally act at stages in the catalytic cycle of the enzyme where both DNA strands are intact and, therefore by definition they are not expected to cause any DNA breakage [2]. While topo II poisons have been thoroughly studied for their mechanism(s) of interference with topo II – some of the more potent and widely used anticancer drugs belong to this category – much less is known about catalytic

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inhibitors and their relationship with topo II, or with respect to their clinical use [3]. Nonetheless, aclarubicin (ACLA) represents an exception in that it is a topo II catalytic inhibitor that has been successfully used in clinical oncology practice. This intercalative antibiotic belongs to the class of anthracyclines, and efficiently binds to DNA, which in turn results in an inhibition of topo II to access the DNA [4–6], i.e. ACLA is a DNA intercalator that indirectly interferes with topo II. On the other hand, besides its role as a topo II catalytic inhibitor, an interesting feature of ACLA is its reported concomitant action as a topo I poison through stabilization of the topo I-DNA cleavable complex [7–10].

This dual mechanism of action of this antibiotic, involving both topo II (catalytic inhibition) and topo I (poisoning) makes this drug an interesting choice for the assessment of the relative importance of either enzyme in DNA function and of the deleterious consequences of topoisomerase dysfunction as a whole. Besides, the dual topo I poisoning-topo II catalytic inhibition exerted by ACLA contrasts with that of other agents acting in a dual fashion, with either a poison–poison [7,11–13] or catalytic–catalytic [10] anti-DNA topoisomerase mechanism.

The early notion that catalytic inhibitors of topo II do not induce DNA breaks through stabilization of the cleavable complex [2,14], because they interfere with the cycle of the enzyme when both DNA strands are intact, has been challenged recently. Reports on e.g., the bis-dioxopiperazine ICRF-193 described the induction of chromosomal aberrations [15,16] as well as DNA strand breaks [17–19], raising questions as to the existence of 'clean' catalytic inhibitors of topo II that act on the enzyme without concomitantly inducing DNA damage, either directly or indirectly. Focusing on ACLA, even though studies on this intercalative antibiotic are scarce, its ability to induce DNA damage in two human myeloid cell lines has been measured by use of the comet assay and reported to correlate with the induction of apoptosis [6].

The sensitivity of radiosensitive mammalian cell lines to topoisomerase poisons was shown to be similar to that observed in response to radiation damage [20–23]. In the case of ACLA, however, taking into account the dual nature of this drug as topo II catalytic inhibitor as well as topo I poison, the picture that arises is somewhat confusing. In the present report, we have evaluated the cytotoxic and/or genotoxic activity of ACLA in two cultured Chinese hamster lung fibroblast cell lines, namely the parental line V79, which repairs DNA radiation damage normally, and its radiosensitive mutant *irs-2* [21], to see whether the effects of ACLA are different between these two cell lines.

2. Materials and methods

2.1. Cells and culture conditions

The parental lung fibroblast Chinese hamster cell line V79 was purchased from the American Type Culture Collection (ATCC), USA. The radiosensitive mutant *irs-2* was kindly provided by Dr. John Thacker (Medical Research Council, Harwell, UK). Cells were routinely maintained as monolayer 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 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. Cell viability

2.2.1. Sulforhodamine B (SRB) assay

V79 and *irs-2* cells in the 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 topo II inhibitor ACLA. The concentration range tested (0.006 to 0.37 μM) was prepared in tissue culture medium from a 1 mM ACLA stock solution.

Following the recommendations of the National Cancer Institute (USA), the analysis of cytotoxic effects induced by ACLA was conducted by use of a cell growth test, the SRB assay, as described previously [24,25]. Briefly, 50 μl/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 proteins and to 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/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/well of 10 mM unbuffered Tris base by shaking. The optical density was read at 492 nm using a microtitre plate reader (ELISA). Similarly, cells from both lines were treated with different doses (0.005–2.5 μM) of the bisdioxopiperazine ICRF-193 in order to assess the relative importance for cytotoxicity of inhibition of topo II catalytic activity compared with poisoning of topo I. Each type of experiment was independently performed in triplicate.

2.2.2. Clonogenic assay

V79 and *irs-2* cells in the exponential growth phase were trypsinized at 37 °C for 5 min, and pipetted five times to remove cell clumps in order to obtain a single-cell suspension. The cells were then counted and seeded into 60 mm tissue culture dishes at various densities, i.e. roughly 500 cells/dish of treated and untreated V79 cells, and 1000 cells/dish of treated and 500 cells/dish of untreated *irs-2* cells. Each dish contained 5 ml MEM, and the experiment was repeated three times.

The cells were incubated for 3 h in the presence of the DNA topo II inhibitor ACLA, diluted in 5 ml tissue culture medium. The concentration range tested was from 0.12 to 1.8 μM. Cells were rinsed twice with MEM and immediately allowed to grow at 37 °C for 15 days into visible colonies, in order to check the clonogenic viability. Control or treated cells were fixed with methanol and stained with Giemsa, and colonies containing more than 50 cells were counted. Four repeat experiments were carried out with exponentially growing V79 and *irs-2*.

2.3. Preparation of nuclear extracts

Exponentially growing V79 and *irs-2* cells were incubated for 3 h in the presence of different concentrations ranging from 0.006 to 2.4 μM of the topo II inhibitor ACLA. After the treatment, the cells were processed to obtain extracts of nuclear proteins, while untreated control cells were also sampled in parallel for comparison. The procedure was basically that described by Heartlein et al. [26]. 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 a 15 min incubation at 0 °C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, 50 mM Tris–HCl pH 7.5, 10 mM β-mercaptoethanol, and 1 mM PMSF were added. The suspension was incubated for a further 40 min 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 [27]. 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 with a TopoGen (Columbus, OH, USA) assay kit based upon decatenation of kinetoplast DNA (kDNA). An amount of 100 ng of nuclear extract protein from either control or ACLA-treated V79 or *irs-2* cells was assayed for topo II catalytic activity. Reaction products were resolved by agarose-gel electrophoresis of DNA. After 40 min incubation at 37 °C, the samples were loaded onto 1% agarose gels and subjected to electrophoresis for 2.5 h at 100 V. Finally, gels were stained with 0.5 μg/ml ethidium bromide, destained (30 min) in distilled water and photographed using a standard photodyne set.

2.5. Comet Assay

V79 and *irs-2* cells were treated for 3 h with two selected concentrations (1.2 and 2.4 μM) of ACLA. Positive controls of both cell lines were obtained by inducing DNA damage through irradiation of the exponentially growing cells with 5 Gy of X-rays using a X-ray machine (Philips MG 103/2.25 system, Germany, 100 KVp, 15 mA, dose-rate 1 Gy/min). To determine the initial DNA damage cells were irradiated

on ice. The comet assay was basically performed according to the original protocol of Singh et al. [28], with some modifications carried out in our laboratory as reported elsewhere [19].

2.6. Micronucleus assay

V79 and *irs-2* cells in the exponential growth phase were incubated for 3 h in the presence of the DNA topo II inhibitor ACLA, diluted in tissue culture medium. The concentration range tested was 0.06–0.3 μM. After treatment, cells were rinsed twice with MEM and cytochalasin B (Cyt B, Sigma; from a 2.0 mg/ml stock solution in DMSO, stored at −80 °C) diluted with PBS was immediately added to the cell cultures at a final concentration of 3.0 μg/ml. After recovery in Cyt B for 36 h, fixation was according to the standard cytological procedure, i.e., with a methanol–acetic acid solution (3:1). Cytological preparations were made by dropping cells onto wet slides and staining with Giemsa.

Two thousand binucleated cells were scored blind for micronucleus frequency in each treatment by different observers. A one-tailed students' *t*-test was used to determine if the number of micronuclei observed in cells treated with ACLA was significantly different from that found in untreated control cells. As reported above for the SRB assay, which included a control with the topo II catalytic inhibitor ICRF-193, micronuclei induced by different doses of the topo I poison camptothecin were tested for comparison with ACLA, in order to discriminate between topo II catalytic inhibition and topo I poisoning as the ACLA activity responsible for chromosome damage.

3. Results

3.1. Inhibition of Topo II catalytic activity by ACLA

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 the intercalative anthracycine ACLA. As can be seen in Fig. 1, in the absence of any drug treatment, topo II

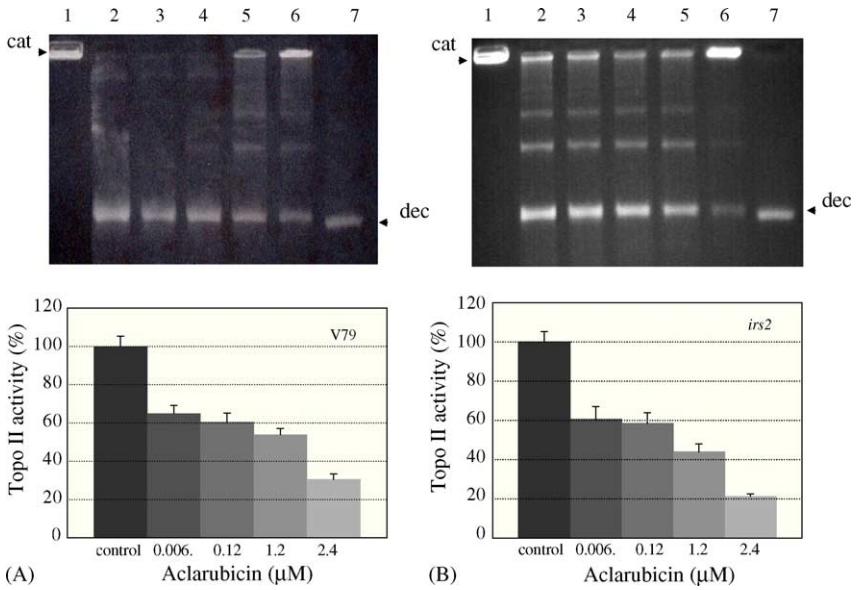

Fig. 1. Effectiveness of different doses of ACLA, ranging from 0.006 to 2.4 μM to inhibit the topo II catalytic activity. Nuclear extracts from V79 (A) and *irs-2* cells (B) were obtained as described in Section 2 and their ability to decatenate catenated kinetoplast DNA was assayed by DNA gel electrophoresis. Lane 1: marker catenated (cat) kinetoplast DNA; lane 2: control cells not treated with ACLA; lanes 3–6 treated with increasing concentrations of ACLA (0.006, 0.12, 1.2, and 2.4 μM, respectively); lane 7: decatenated (dec) DNA. Below, the respective densitometric profiles are shown. The loss of topo II catalytic activity in ACLA-treated cells was in all cases significant compared with non-treated cells ($P < 0.001$; Student's *t*-test).

activity recovered in nuclear extracts from both V79 and *irs-2* was able to efficiently decatenate the catenated DNA substrate as shown by the release of closed minicircles. When the possible inhibition by increasing concentrations of ACLA was tested, a dose-dependent inhibitory effect was seen with both cell lines, as shown by a progressive increase in the amount of catenated DNA substrate remaining in the wells (Fig. 1). On the other hand, image densitometry showed no significant differences between the repair-proficient parental V79 cells and the radiosensitive *irs-2* mutant (Fig. 1) with respect to the effect of ACLA in the dose range 0.006–2.4 μM.

While all ACLA concentrations tested inhibited topo II to different degrees, doses of 1.2 and 2.4 μM ACLA were most effective in the prevention of the release of DNA minicircles from the catenated substrate (kDNA) by topo II, showing a three- to five-fold reduction in enzyme activity (Fig. 1). On the basis of these results, dose intervals were selected for the experiments assessing cytotoxic or genotoxic effects of ACLA in the two cell lines (see below).

3.2. Cytotoxicity of ACLA

To compare the radiosensitive mutant *irs-2* with its parental V79 cell line with respect to their possible differential response to ACLA treatment, two different approaches were chosen. First, the cytotoxicity of ACLA was determined by means of the SRB assay, which measures protein production of the cell as a whole. Fig. 2 shows the results obtained for both cell lines after treatment with a range of concentrations of ACLA previously shown to inhibit the catalytic activity of topo II in a dose-dependent fashion (Fig. 1). The results of the SRB assay clearly indicate a dose-dependent cytotoxic effect of ACLA in both cell lines, but for any of the concentrations tested the drug appears to be more cytotoxic for the radiosensitive mutant *irs-2* than for the repair-proficient V79 cell line (Fig. 2). When this result on cell viability was compared with that observed for the bisdioxopiperazine ICRF-193, considered as a topo II catalytic inhibitor, a very different pattern was found. Both at doses equimolar and higher than that of ACLA, only a maximum of 20% viability was lost, while this was up to 80% after ACLA treatment. An additional interesting feature observed in this study was that no difference was apparent between the two cell

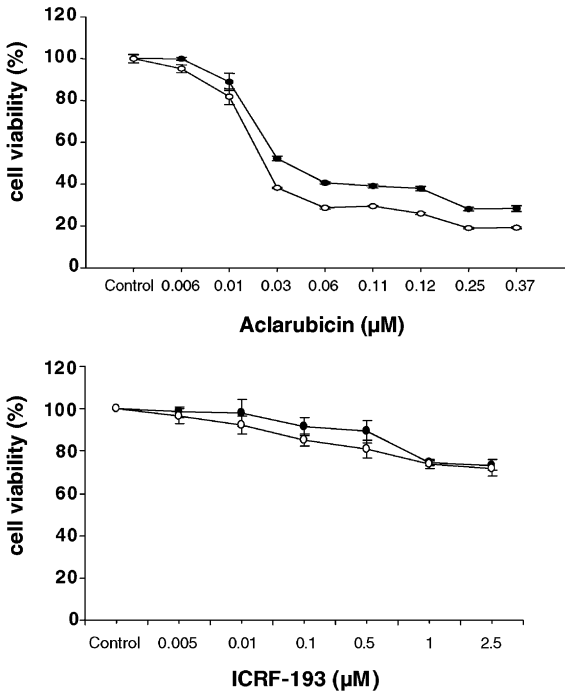

Fig. 2. Comparative effects of different concentrations of the anti-topo II ACLA to suppress cell viability in the parental cell line V79 (●) and its radiosensitive cell mutant *irs-2* (◯) as shown by the SRB assay. Bars indicate standard deviation from three independent experiments. Statistical analysis (Student's *t*-test) shows a significant difference ($P = 0.02$) between the two cell lines only for the highest two doses of ACLA. Effectiveness of the topo II catalytic inhibitor ICRF-193 on the two cell lines is also shown as a control, to assess the relative importance of inhibition of the catalytic activity of topo II by ACLA.

lines after treatment with ICRF-193, in contrast to what was found for ACLA. This latter result seems to indicate that topo II catalytic inhibition is not the main factor responsible for the cytotoxic effects induced by ACLA.

Besides the SRB assay, in order to test the effectiveness of ACLA to induce proliferative death in the cells after a prolonged treatment, a clonogenic study was carried out. Experiments were performed at least three times for every experimental point, and Fig. 3 shows the data on colony-forming ability of both V79 and *irs-2*. In good agreement with the SRB data, ACLA has a dose-dependent negative effect on the proliferative rate of both cell lines, but the reduction in surviving colonies was higher in the radiosensitive *irs-2* cells for most of the ACLA doses tested.

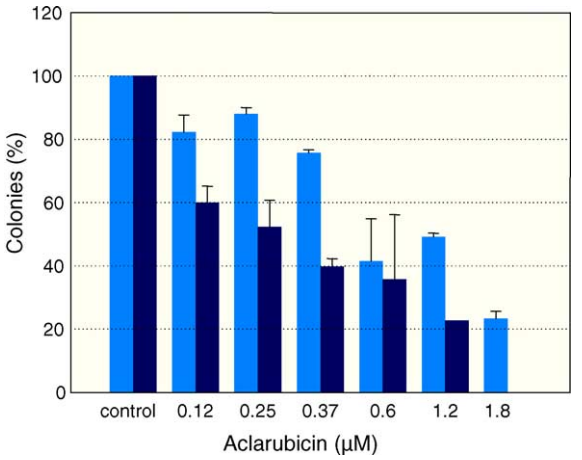

Fig. 3. Colony-forming ability in V79 (■) and *irs-2* (■) cells treated with ACLA. Cells were exposed for 3 h to 0.12–1.8 μM ACLA. After 15 days, the number of surviving colonies was counted. These experiments were performed in triplicate. Bars indicate standard deviation of the mean.

3.3. DNA strand breaks induced by ACLA

The alkaline single-cell gel electrophoresis (SCGE) or 'comet assay', which provides a measure of both single- and double-strand breaks in DNA as well as alkali-labile sites, was the method of choice to investigate DNA-damage induction. V79 and *irs-2* cells treated with ACLA for 3 h showed that the anthracycline treatment is effective in inducing DNA damage (Fig. 4). While the tail-moment values did not reach the level found in the positive control irradiated with 5 Gy of X-rays, mainly for the radiosensitive mutant *irs-2*, ACLA acts as a DNA-damaging agent in both cell lines (Fig. 4). These results are in good agreement with those reported by Gieseler et al. [6] on the ability of ACLA to induce DNA damage in two human myeloid cell lines, as measured with the comet assay.

Comparing the parental V79 cells and the radiosensitive mutant *irs-2*, we have not observed any significant difference, as shown by a very similar profile of the tail moment distribution in both cell lines for the doses of ACLA assayed, immediately after the 3 h-treatment (Fig. 4).

3.4. Micronuclei in binucleated cells

In order to assess the behaviour of V79 and *irs-2* cell lines with respect to their recovery from DNA damage induced by ACLA (see above), we analysed micronucleus formation in Cyt-B-induced binucleated cells. To account for the delay observed after a 3 h treatment with ACLA, a recovery time of 36 h in the presence of Cyt-B was allowed for both cell lines, and the results are shown in Fig. 5. For all the doses that were compatible with progress through the cell cycle (dose range 0.06–0.3 μM), the *irs-2* cells showed a statistically significant increase in the yield of micronuclei, as

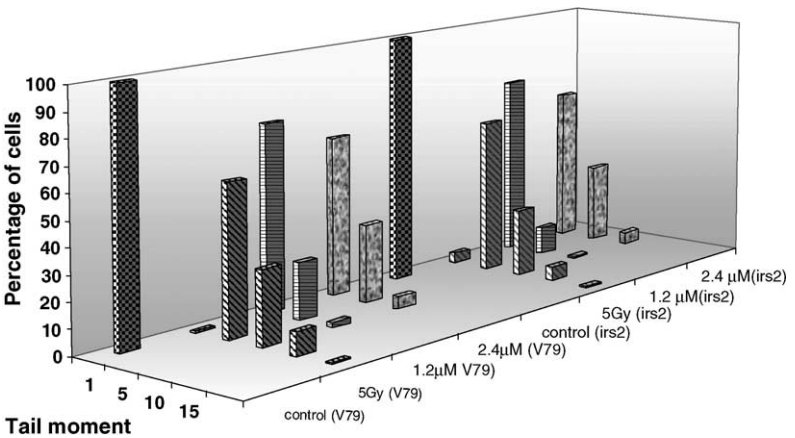

Fig. 4. Effectiveness of different concentrations of the topoisomerase dual inhibitor ACLA to induce DNA damage in V79 and *irs-2* cells, as shown by the comet assay. Cells exposed to 5 Gy of X-rays were used as a positive control. Data from three independent experiments (50 comets were measured per experimental point in each experiment). Note the dose-dependent increase in tail moments, as compared to non-treated controls ($P < 0.0001$; Student's *t*-test).

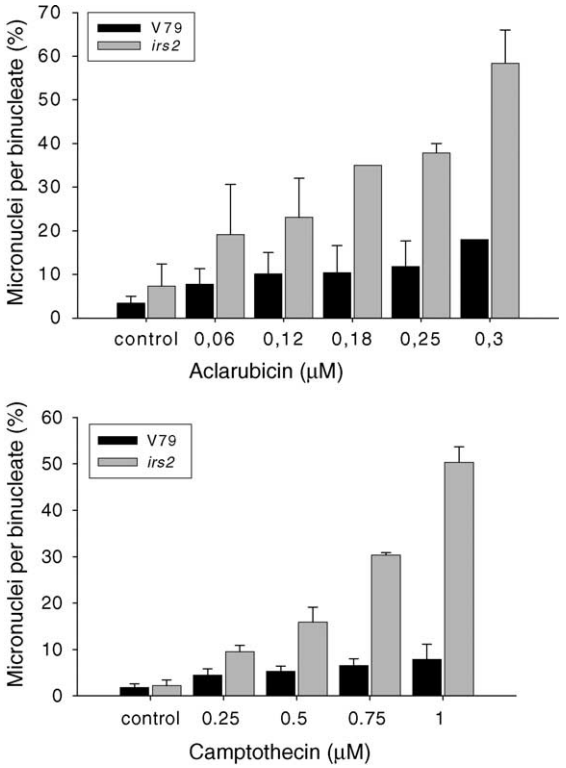

Fig. 5. Induction of micronuclei in V79 (■) and *irs-2* (■) cells by ACLA. Cells were treated for 3 h with increasing concentrations of ACLA (0.06, 0.12, 0.18, 0.25 and 0.3 μM), followed by a post-treatment period (36 h repair period) in fresh medium. Comparative effects of different doses of the topo I poison camptothecin are also presented, to assess the relative importance of poisoning of topo I by ACLA, one of its two functions, for chromosome breakage. Data represent the average of three independent experiments. Data are given as the mean ± S.D. The difference between the two cell lines in their response to ACLA and camptothecin was statistically significant ($P < 0.001$), the induction of micronuclei being consistently higher in the radiosensitive mutant *irs-2* than in the parental V79.

compared with that observed in the parental V79 cell line (Fig. 5). This observation is in contrast with the apparent lack of a significant difference immediately after ACLA treatment (see above, Fig. 4), and seems to support the hypothesis of a clear difference between the cell lines with regards to processing of ACLA-induced damage in a way that is similar to that reported for radiation damage, provided that sufficient recovery time is allowed.

As to the relative importance of topo I poisoning for chromosome damage induced by ACLA, a control experiment using the topo I poison camptothecin was carried out. As can be seen in Fig. 5, camptothecin treatment resulted in a very similar pattern of induced micronuclei, again with a higher value found in *irs-2* cells compared with parental V79 cells for any given dose. This seems to indicate that poisoning of topo I plays the major role compared with catalytic inhibition of topo II for genotoxic as well as cytotoxic effects of ACLA.

4. Discussion

The anthracycline ACLA is one of the most successful and widely used topo II catalytic inhibitors to treat acute leukaemias, lymphomas, and a variety of solid tumors. At the molecular level it indirectly interferes with topo II, but it probably targets other enzymes as well, given its potent intercalation into DNA. It has been reported that the drug acts by preventing the binding of topo II to DNA, as a consequence of its efficient intercalation, thus modifying dramatically the double-stranded molecule [29]. It is widely accepted that catalytic inhibitors acting in this fashion are generally able to abrogate DNA damage and cytotoxicity caused by topo II poisons such as etoposide or amsacrine [3]. By their antagonistic effect, the formation of cleavable complexes topo II-DNA is very much decreased through a shortage in the available target of the poison.

A question of great importance is whether an agent targeting topo II kills the cells because of the generation of DNA damage, or because an enzyme necessary for chromosome separation (and perhaps other processes) is inhibited. A secondary question that has not been completely answered is whether inhibition of topo II catalytic activity in mammalian cells can lead to DNA damage as a secondary consequence. Since ACLA is a dual topoisomerase inhibitor that behaves also as a topo I poison able to stabilize topo I-DNA cleavable complexes with the subsequent production of DNA strand breaks [1], its anti-cancer properties have been ascribed to this latter activity, in a similar fashion to that reported for camptothecin [30–32]. Concerning this, the results presented here seem to support this hypothesis, taking into account that ACLA has been shown to induce micronuclei similar to the topo I poison camptothecin, while the cytotoxicity of ACLA is much higher than that of the topo II catalytic inhibitor ICRF-193.

In the present investigation, we have also studied the ability of different doses of ACLA to inhibit the topo II catalytic activity, and compared the cytotoxic and genotoxic effect of this drug in two cultured Chinese hamster lung fibroblast cell lines with different radiosensitivity, namely the repair-proficient parental line V79, and its radiosensitive mutant *irs-2* [33,34]. The interest of this comparison arises from the general observation that radiosensitive mammalian cell lines also appear to be hypersensitive to topoisomerase poisons [21–23,35,36].

The mutant *irs-2* was isolated from Chinese hamster V79 on the basis of its hypersensitivity (two- to three-fold) to cell inactivation by X-rays. These cells are phenotypically similar to those of the human cancer-prone syndrome ataxia telangiectasia (A–T) in that both show radioresistant DNA synthesis [37]. Nevertheless, the molecular basis of the radiosensitivity of *irs-2* cells has not been unequivocally determined. It has been reported that *irs-2*, which belongs to the X-ray repair cross-complementation (XRCC) group, is also highly sensitive to the topo I poison camptothecin [21,36], while it shows little or no increased sensitivity to topo II inhibitors [36]. Interestingly, like AT cells and hamster *irs-2* cells, Nijmegen breakage syndrome (NBS) cells are X-ray sensitive (two-fold), display radioresistant DNA synthesis and have been reported to be about three-fold more sensitive to camptothecin [38] than normal cells.

As to the nature of *irs-2* hypersensitivity no clear conclusion has been reached so far, as the level of topo I activity was similar to that found in the parental V79 cells, and no difference was observed in either the number of breaks induced by camptothecin or the rate of reversal following drug removal [36]. Despite this lack of correlation between any of the parameters analysed and cell sensitivity to camptothecin, new Chinese hamster cell mutants recently isolated on the basis of their sensitivity to the drug appear to be members of the *irs-2* (XRCC8) complementation group. This stresses the importance of the role played by XRCC8 in DNA repair, the cell cycle as a whole and in DNA damage response mechanisms [39].

With respect to basal topo II catalytic activity or the response to ACLA in terms of enzyme inhibition, the radiosensitive mutant *irs-2* and its parental line V79 are rather similar. This observation seems in good agreement with the lack of differences in topo I activity between V79 and *irs-2* reported by Jones et al. [36], and also appears to support the idea that differences between the two cell lines in their sensitivity to topo I or topo II inhibitors cannot be ascribed to any enzyme abnormality.

The above notwithstanding, a higher cytotoxicity in ACLA-treated *irs-2* cells compared with V79 was observed in the SRB assay, which measures the whole protein production of the cell, and was also shown by the colony-forming ability of the cells in the presence of the drug. When these observations concerning the differential cytotoxic effects of ACLA on the radiosensitive AT-like mutant *irs-2* and the parental line V79 were compared with the data on the production of DNA strand breaks measured by use of the ‘comet’ assay shortly after ACLA treatment, no significant difference in overall DNA damage was observed. Nevertheless, after a long recovery time the picture came out completely different. Indeed, the induction of micronuclei in binucleated cells showed that the anthracycline treatment was more effective in the *irs-2* cell line than in V79 cells. This result seems to be in good agreement with our data on differences in cytotoxicity induced by ACLA in the two cell lines. Concerning the reported dual role of ACLA, as stated above, we think that the higher yield of DNA damage detected in *irs-2* cells by the micronucleus test compared with the parental line V79 is likely to be derived from the topo I poisoning activity of ACLA [7–10].

Induction of DNA damage as determined by the ‘comet assay’ has been reported earlier for this anthracycline [6]. Interestingly, only 25–40% of the exposed cells (3 h ACLA exposure time) showed visible DNA damage, and it was concluded that ACLA-induced DNA strand breakage is likely to occur during S phase, maybe in connection with topoisomerase activity in relaxing supercoils ahead of the replication fork [6]. Also, regardless of the concentration of ACLA used, roughly 60–70% of the cells show no DNA damage after a 3 h-treatment with the topoisomerase inhibitor.

Concerning the fate of DNA lesions induced by ACLA, however, our results seem to be at variance with those reported by Jones et al. [36] using the topo I inhibitor camptothecin. These authors reported no difference between V79 and *irs-2* in either the number of breaks induced by the topo I poison or the rate of their reversal following drug removal. Contrasting with

this latter conclusion, we have found clear-cut cell line-dependent differences in recovery after ACLA damage, as shown by the micronucleus frequency observed after a prolonged post-treatment time.

Taken as a whole, our results seem to lend support to the general view that the radiosensitivity of cells parallels their sensitivity to topoisomerase poisons, and that poisoning of topo I, one of the dual functions of ACLA, is likely to be responsible for DNA strand break induction by this chemotherapeutic drug.

Acknowledgement

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