ELSEVIER

Mutation Research 515 (2002) 171–180

Genetic Toxicology and Environmental Mutagenesis

www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

A comparative study of genotoxic effects of anti-topoisomerase II drugs ICRF-193 and bufalin in Chinese hamster ovary cells

Nuria Pastor, Inmaculada Domínguez, Santiago Mateos, Felipe Cortés*

Department of Cellular Biology, Faculty of Biology, University of Seville, Avda. Reina Mercedes No. 6, 41012 Seville, Spain

Received 28 September 2001; received in revised form 8 January 2002; accepted 9 January 2002

Abstract

With the ultimate purpose of testing the existence of possible differences in the effectiveness of the topoisomerase II catalytic inhibitor ICRF-193 (a bisdioxopiperazine) and the enzyme suppressor bufalin (a bufadienolide from toad venom) we have carried out a series of experiments aimed at inducing cytotoxicity as well as DNA and chromosome damage in transformed CHO cells. In order to assess any possible influence of DNA repair capacity of the treated cells on the final outcome, we have made use of the repair-defective CHO mutant EM9, which shows a defect in DNA single- and double-strand breaks repair for comparison with its repair-proficient parental line AA8.

Our results seem to indicate that, while both ICRF-193 and bufalin suppress cell growth and result in a clear inhibition of topoisomerase II catalytic activity, only ICRF-193 has been shown as able to induce both chromosome and DNA damage, with a more pronounced effect in the CHO mutant EM9 than in the repair-proficient line AA8. © 2002 Published by Elsevier Science B.V.

Keywords: Topoisomerase II inhibitors; Cytotoxicity; DNA damage

1. Introduction

Apart from their fundamental roles in virtually every aspect of DNA metabolism through the performance of topological changes needed for replication, transcription, recombination and segregation of daughter molecules [1], DNA topoisomerases (topos) represent nowadays a major focus of research for cancer chemotherapy [2].

On normal functioning, topo I relaxes supercoiled DNA that generates during replication and transcription by forming a covalent bond with the 3′-terminus of a DNA single-strand break [3], while the dimeric topo II is able to play its unique role in decatenation and unknotting of entangled DNA through the formation of DNA double-strand breaks with the enzyme protomers covalently bound to the 5′-terminus. In this latter case, the opening of a protein gate allows the passage of intact double-stranded DNA [4].

The 'classical' topoisomerase poisons that represent some of the most efficient and widely prescribed anticancer drugs currently utilized for the treatment of human neoplasms, act in an insidious fashion and kill cells by stabilization of the otherwise fleeting intermediates so-called cleavable complexes, made up of the ternary structure drug–enzyme–DNA. On interference with replication and/or transcription, cell death mechanisms are somehow triggered [5].

While topo I is the specific target for only a limited group of drugs acting as poisons of the enzyme, such as camptothecins and derivatives [5], topo II is the primary target of poisoning by an increasing number

* Corresponding author. Tel.: +34-95-4557039; fax: +34-95-4610261.
E-mail address: cortes@us.es (F. Cortés).

1383-5718/02/$ – see front matter © 2002 Published by Elsevier Science B.V.
PII: S1383-5718(02)00011-6

of cytotoxic drugs of diverse nature currently available for the clinical treatment of human cancers [2,6–8].

The list of clinically important topo II targetting antitumor drugs include anthracyclines, e.g. adriamycin and daunorubicin, epipodophyllotoxins, e.g. etoposide and teniposide, anthracenedione, e.g. mitoxantrone, and aminoacridines, e.g. *m*-AMSA [9]. Etoposide (VP-16), for instance, is one of the most commonly prescribed anticancer drugs that is front-line therapy for small-cell lung cancer as well as a variety of other malignancies, such as leukemias, lymphomas, and germ-line neoplasms [10,11]. Since the concentration of topo II is usually elevated in rapidly proliferating or transformed cells [12], clinically agressive cancers appear to be the most responsive to these drugs.

Besides the cleavable complexes-stabilizing topo II poisons, a separate group of drugs have been more recently reported to act as 'true' catalytic inhibitors [9]. These chemicals do interfere with the catalytic cycle of the enzyme, but unlike topo II poisons they lack the ability to stabilize the cleavable complex and are considered not to induce DNA strand breaks [13,14]. These non-classical drugs have attracted clinical interest because they appear to circumvent the at-MDR phenotype [15] and may operate as antagonists to topo II poisons. They include aclarubicin, fostriecin, merbarone, suramin, quinobenoxazine, bisdioxopiperazines (ICRF-154, etc.), chloroquine and novobiocin [2,9].

Given their possible use in rescue regimes in combination with topo II poisons as a new strategy to improve tumor selectivity [16], catalytic inhibitors of the enzyme have drawn a lot of interest in the last years. Furthermore, this class of inhibitors have opened a new field in what concerns to the possibility to study the physiology of topoisomerases, overcoming the difficulties encountered earlier when only DNA-damaging poisons were available.

We have carried out a comparison of the cytotoxic and genotoxic effects of two topo II-targeted drugs that do not act as cleavable complex stabilizers, namely the bis(dioxopiperazine) ICRF-193, that can be considered as the most potent topo II catalytic inhibitor, and bufalin, one of the components of the bufadienolides in the traditional Chinese medicine. DNA repair-proficient AA8 Chinese hamster cells as well as repair-defective EM9 have been treated with these anti-topo II drugs, in order to assess any possible influence of DNA repair on the outcome of treatments.

Our results seem to indicate that important differences do exist between both drugs as to their possible effects, while the cell capability to efficiently carry out repair does not seem to be a decisive factor, so contrasting with that reported for topoisomerase poisons [17].

2. Materials and methods

2.1. Culture conditions

The parental cell line AA8 and mutant EM9 were grown as monolayers in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2×10^{-3} M L-glutamine and the antibiotics penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were grown in the dark at 37 °C in a 5% CO_2 atmosphere.

2.2. Drugs

ICRF-193 and bufalin were obtained from Biomol (No. 53618, Germany) and Sigma, respectively, they were dissolved in dimethyl sulfoxide (DMSO) (made up fresh for each experiment) and directly added to the culture medium.

2.3. Growth-inhibition assay

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). After 24 h, to allow cell recovery, they were incubated for 48 h in the presence of the DNA topoisomerase II inhibitory agents, diluted in tissue culture medium (100 μl). The doses range tested was from 5×10^{-7} to 5×10^{-4} M for bufalin and from 10^{-8} to 25×10^{-6} M for ICRF-193.

For growth inhibition studies, the sulforhodamine B (SRB) assay was used as described previously [18,19]. 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 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. Then the plates were air-dried. The stained protein was solubilised 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 experiment was performed in triplicate and carried out three times or more independently.

2.4. Preparation of nuclear extracts

Topo II activity in nuclear extracts from AA8 and EM9 cells that had been treated with different doses of bufalin and ICRF-193 for 22 h were obtained as described by Heartlein et al. [20]. Approximately, 10×10^6 cells were suspended in 1 ml of 0.32 M sucrose, 0.01 M Tris–HCl pH 7.5, 0.05 M $MgCl_2$ and 1% Triton X-100 and thoroughly vortexed to lyse the cells. Nuclear pellets were obtained by centrifugation at $1800 \times g$ (Eppendorf centrifuge), for 5 min at 4 °C. Nuclei were then washed in 1 ml of nucleus wash buffer (5×10^{-3} M potassium phosphate buffer, pH 7.5, 10^{-3} M phenylmethyl sulfonyl fluoride (PMSF), 10^{-3} M β-mercaptoethanol and 0.5×10^{-3} M dithiothreitol (DTT)). The nuclei were then pelleted as described above and resuspended in 50 μl of nucleus wash buffer, and 50 μl of 4×10^{-3} M EDTA was added. Following incubation at 0 °C for 15 min, the nuclei were lysed by adding 100 μl of 2 M NaCl, 20×10^{-3} M Tris–HCl pH 7.5, 10^{-2} M β-mercaptoethanol and 10^{-3} M PMSF. Following a 15 min incubation at 0 °C, 50 μl of 18% polyethylene glycol (PEG-6000) in 1 M NaCl, 50×10^{-3} M Tris–HCl pH 7.5, 10^{-2} M β-mercaptoethanol, and 10^{-3} M PMSF was added. The suspension was incubated for a further 40 min period at 0 °C. The supernatant from a 30 min centrifugation at $11{,}200 \times g$ at 4 °C was then collected. Total protein concentration in each extract [21] was determined in a Beckman DU-64 spectrophotometer by the Bio-Rad protein assay (Bio-Rad Laboratories) and extracts were kept at −80 °C for no longer than a month.

2.5. Topoisomerase II activity in nuclear extracts

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

2.6. Comet assay

AA8 and EM9 cells were treated with increasing doses of up to 5×10^{-4} M for bufalin during 3, 14 and 22 h and 25×10^{-6} M for ICRF-193 only for 3 h.

The assay was basically performed according to the original protocols of Singh et al. [22]. 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 they were used.

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 eight minutes 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 [23]. 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).

Images of 50 randomly selected cells were analysed from each sample. The slides were examined at 200× magnification using a 20× objective on a fluorescence microscope OLYMPUS Vanox AHBT3, excitation filter of 550 nm and barrier filter of 590 nm. Measurements were made by image analysis CASys software (Synoptics, Ltd, image processing systems, UK), and the parameter chosen was the tail moment (tail length X tail intensity or percent migrated DNA) [24].

2.7. Chromosomal analysis

Exponentially growing AA8 and EM9 cells were treated with different doses of the topoisomerase II inhibitors ICRF-193 and bufalin. ICRF-193 was added to the cultures at concentrations of 10^{-9}, 5×10^{-9}, 10^{-8}, 5×10^{-8} and 10^{-7} M and the cells were in the presence of this inhibitor for 22 h. After that cells were washed and kept in fresh medium for 5 h to allow them to recover. Two sets of cultures were used for bufalin treatment, each of them receiving 10^{-6}, 10^{-5}, $10^{-4}$and 5×10^{-4} M of the inhibitor. One set of them was in the presence of bufalin for 22 h, and subsequently the cultures were washed and maintained in fresh medium for 5 h to let them to recover. The other set of cultures received the treatments for 22 h without any recovery. For both cell lines, cultures which did not receive any treatment served as controls. Colcemid (2×10^{-7} M) was added for 3 h to all the cultures for metaphase arrest. Two independent experiments were carried out in each case.

The flasks were shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 0.075 M KCl for 2 min, fixed in methanol/acetic acid (3:1) and dropped onto clean glass microscope slides. The slides were stained with 3% Giemsa and used for chromosome aberration scoring. One hundred metaphases were scored blind for each treatment from two independent experiments.

3. Results

3.1. Growth inhibition (SRB) induced by ICRF-193 and bufalin

Treatment of parental AA8 and repair-deficient EM9 CHO cells with the bis-dioxopiperazine ICRF-193 at concentrations above 10^{-7} M caused a decrease in cell viability as shown by the SRB assay (Fig. 1A). On the other hand, a higher sensitivity, as estimated by an increased loss of cell survival was observed for the mutant EM9 cells ($P < 0.05$; Student's *t*-test) as compared to the parental repair-proficient AA8 cell line (Fig. 1A).

When we examined the same question for bufalin, it became apparent that this drug is also capable of inducing a dramatic loss of cell viability, more evident at doses higher than 10^{-5} M (Fig. 1B). Contrasting with that observed for ICRF-193, no difference between the parental AA8 and the repair-defective mutant EM9 cell lines was shown by our SRB growth inhibition assay (Fig. 1B).

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

In good agreement with the observed negative effect of both ICRF-193 and bufalin on cell growth (Fig. 1), it was shown that both drugs were capable of efficiently inhibiting the topo II catalytic activity in nuclear extracts from both AA8 and EM9 cells.

Fig. 2 shows the loss in capacity to decatenate double-stranded catenated kinetoplast DNA (kDNA) as a result of ICRF-193 treatment in both AA8 (Fig. 2A) and EM9 cells (Fig. 2B). As can be seen, for the doses tested a good correspondence seems to exist between the capacity of ICRF-193 to inhibit topo II catalytic activity and the negative effect on cell growth reported above (Fig. 1).

Similar results on the efficiency of bufalin to inhibit topo II catalytic activity were observed for both AA8 (Fig. 3A) and EM9 (Fig. 3B) cell lines. As already observed for ICRF-193, a good correspondence between the doses of bufalin shown as able to result in a loss of cell growth (Fig. 1) and those resulting in a clear inhibition of topo II catalytic activity was observed.

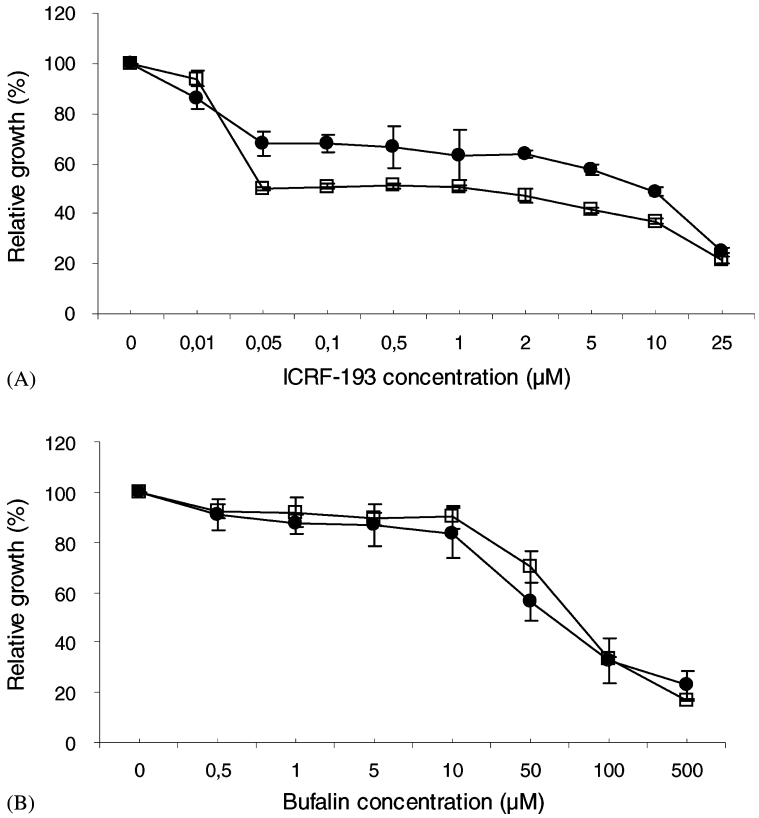

Fig. 1. Effectiveness of different concentrations of the anti-topo II ICRF-193 (A) and bufalin (B) to suppress cell growth in the CHO mutant EM9 (●) and its parental line AA8 (□) as shown by the SRB assay. Bars indicate standard deviation from three independent experiments. Statistical analysis (Student's *t*-test) shows a significant difference ($P = 0.02$) between both cell lines only for ICRF-193.

3.3. DNA damage (Comet assay)

Fig. 4 shows the observations made on the possible DNA damage induced by the bis-dioxopiperazine ICRF-193 on AA8 and mutant EM9 cells as assessed by single cell gel electrophoresis or "Comet assay".

It becomes apparent (Fig. 4) that ICRF-193 treatment for 3 h results in DNA damage, as shown by clearly increased values of the Comet tail moments for both AA8 and EM9 cells. Nevertheless, the mutant repair-deficient EM9 cell line appears as more sensitive to ICRF-193 than its parental line AA8, based upon a higher values of tail moment observed for any given dose of the topo II catalytic inhibitor (Fig. 4A and B, for comparison).

As to the possible ability of bufalin to induce DNA damage, contrasting with that observed for ICRF-193, no increase in tail moment was observed neither in bufalin-treated AA8 nor EM9 nuclei (not shown).

3.4. Chromosomal aberrations

As can be seen in Table 1, again in good agreement with the observations on DNA damage (Comet assay), we also found a dose-dependent clastogenic effect of ICRF-193 for doses of 5×10^{-9} M and higher, while bufalin showed as unable to induce chromosome damage at any of the doses tested (data not shown), regardless of the above reported ability to inhibit topo II catalytic activity.

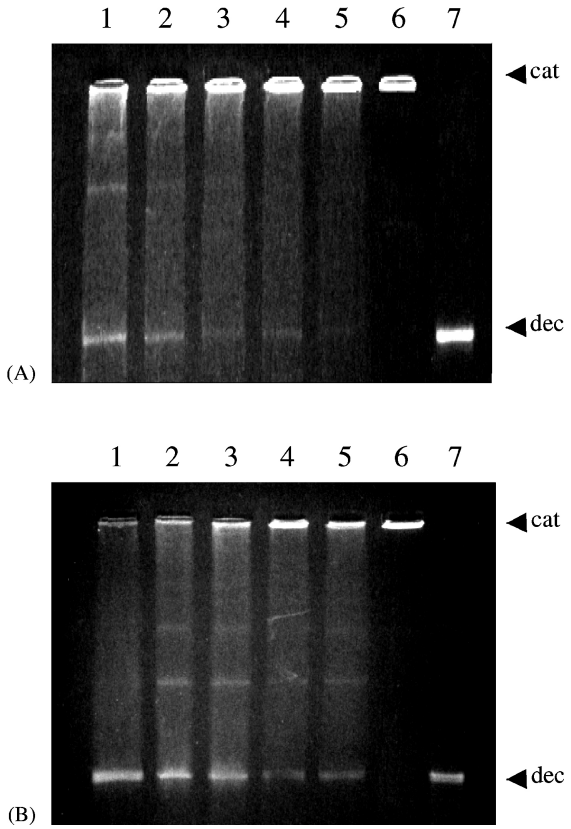

Fig. 2. ICRF-193 treatment and loss of topo II catalytic activity. (A) Capacity to decatenate double-stranded catenated kinetoplast DNA (kDNA) in AA8 and (B) the same for the CHO mutant EM9. For both cell lines, lane 1, control; lane 2, 2×10^{-6} M ICRF-193; lane 3, 5×10^{-6} M; lane 4, 10^{-5} M; lane 5, 25×10^{-6} M; lanes 6 and 7, marker catenated kinetoplast DNA (cat) and decatenated (dec), respectively.

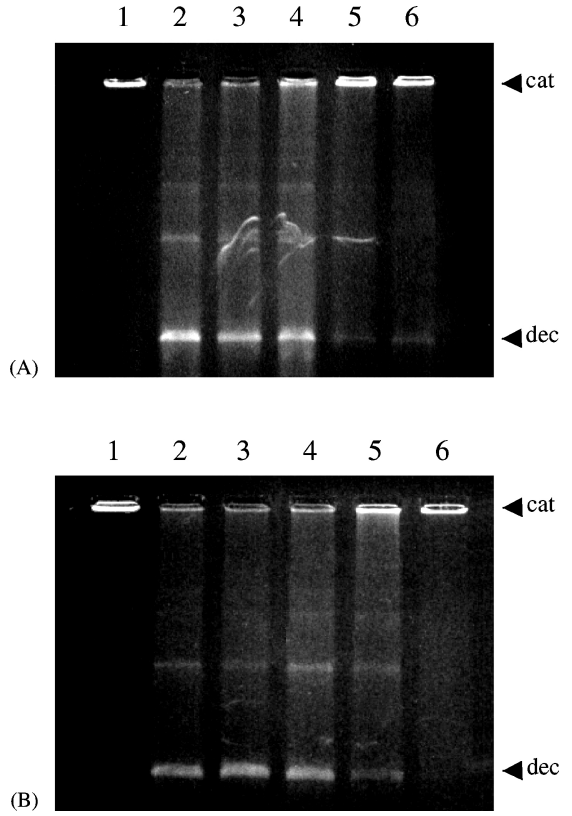

Fig. 3. Effect of the topo II suppressor bufalin on the catalytic activity of topo II in both AA8 (A) and EM9 cell line (B): lane1, marker catenated kinetoplast DNA (cat); lane 2, control; lane 3, 10^{-6} M bufalin; lane 4, 10^{-5} M; lane 5, 10^{-4} M and lane 6, 5×10^{-4} M.

Table 1
Frequency of chromosomal aberrations (CA) in AA8 and EM9 cells treated with different doses of ICRF-193

Dose	Types of chromosomal aberrations													
	AA8							EM9						
	CB	IB	E	D	R	MA	% CA (± S.D.)	CB	IB	E	D	R	MA	% CA (± S.D.)
Control	3	3	0	2	0	0	4.0 (±0.43)	5	10	3	2	0	0	10 (±0.62)
10^{-9} M	4	3	0	2	0	0	4.5 (±0.41)	4	9	1	5	0	1	10 (±0.62)
5×10^{-9} M	3	7	3	1	1	0	7.5 (±0.54)	10	20	3	1	1	1	18 (±0.82)
10^{-8} M	11	20	2	2	2	23	18.5 (±1.03)	8	34	10	11	1	25	32 (±1.10)
5×10^{-8} M	22	88	73	15	9	111	104.5 (±1.99)	32	185	101	32	8	73	179 (±2.62)
10^{-7} M	–	–	–	–	–	–	NS[a]	–	–	–	–	–	–	NS[a]

CB: chromatid breaks, IB: isochromatid breaks; E: exchanges; D: dicentrics; R: rings.; MA: multiple aberrations. This latter were not included in the statistical data. 200 well-spread metaphases per dose were scored.

[a] NS: not scorable.

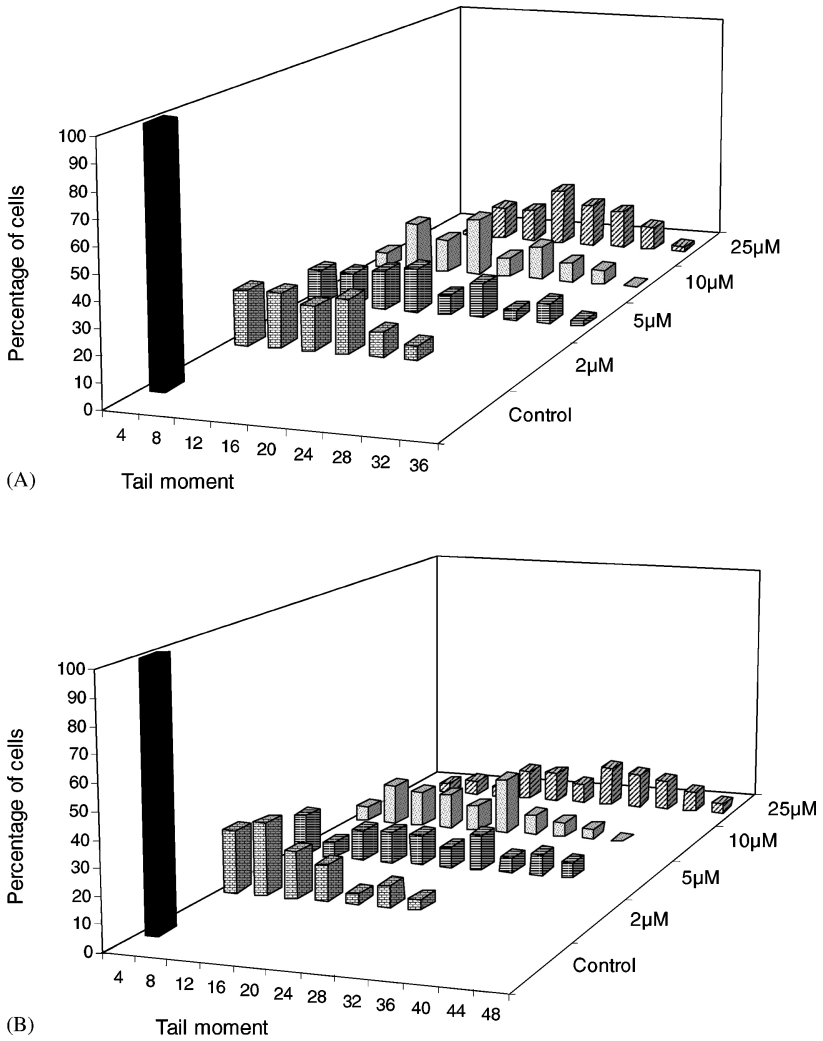

Fig. 4. Effectiveness of different doses of the topo II catalytic inhibitor ICRF-193 to induce DNA damage in AA8 (A) and EM9 (B) cells, as shown by the Comet assay. Observe the dose-dependent increase in tail moments, as compared to non-treated controls ($P < 0.0001$; Student's *t*-test).

4. Discussion

Besides DNA topo II poisons, true catalytic inhibitors of the mammalian enzyme that do not stabilize the cleavable complex have been found more recently in natural and synthetic compounds. This second group of drugs target topo II within the cell and prevent various genetic processes which depend upon a good function of the enzyme, such as DNA replication, and chromosome segregation and condensation. Contrasting with the topo II poisons, however, the manner by which these agents inhibit different steps of the catalytic cycle of the enzyme and the exact mechanism by which they can become cytotoxic through their interference with topo II function are only partly understood [2,9].

The above notwithstanding, catalytic inhibitors of the enzyme are currently being thoroughly investigated, given their possible clinical implications [15,25–27]. On the other hand, consistent results as

to the identity of the in vitro and the actual in vivo target(s) of the bisdioxopiperazine ICRF-193 have been published [28,29].

We have treated transformed CHO cells with two drugs (ICRF-193 and bufalin) reportedly able to exert their action on topo II, apparently through very different molecular mechanisms, with the ultimate purpose of assessing any possible cytotoxic and genotoxic effect. Cell growth was efficiently inhibited by both ICRF-193 and bufalin, at doses shown as able to inhibit topo II catalytic activity in vivo. While in the case of ICRF-193, and in general the bisdioxopiperazines, has been reported that it appears to lock the enzyme in the closed-clamp post-religation step through inhibition of the intrinsic ATPase activity [1,30], the molecular mechanism of bufalin that results in a strongly disminished topo II activity after in vivo treatment, is at present rather poorly understood. What seems clear is that a direct interaction of the drug with topo II can be ruled out, according to our observations in vitro.

Bufalin, one of the prominent components of bufadienolides, in Chinese toad venom, markedly reduces the level and the activity of topo IIα and topo IIβ in human leukemia cells [31] and was reported to induce differentiation in human myeloid leukemia cell lines through an as yet undetermined mechanism [32,33] and was also shown to inhibit solid tumor growth [34]. Bufalin inhibition of endotelial cell proliferation and angiogenesis in vitro has also been recently reported [35]. Most of these effects have been attributed to result from the G2/M phase arrest of cell cycle in bufalin-treated cells [36] that eventually can lead to the triggering of apoptosis [37–40]. Pretreatment of human leukemia HL60 cells with bufalin also resulted in an increase in the inhibitory effect of the anti-tumor drugs cisplatin and all-trans retinoic acid [31].

Our observations on a negative effect of bufalin on cell growth in CHO cells, in a similar fashion to that found for the topo II catalytic inhibitor ICRF-193 adds to the above reports and seem to support the possible usefulness of this compound in cancer therapy. A comparison of the relative effectiveness of either ICRF-193 and bufalin on AA8 (parental) and EM9 (repair-deficient mutant) CHO cells shows that these latter appear as less sensitive to ICRF-193 than AA8, while no difference is observed between both cell lines as to their response to bufalin treatment. An explanation for the difference found between AA8 and EM9 concerning the effectiveness of ICRF-193 to negatively affect cell growth is not at hand, but it seems consistent with our observation on a much higher induction of endoreduplication also in EM9 (unpublished data). Since ICRF-193 in spite of being a non cleavable complex stabilizer induces DNA damage and chromosomal aberrations (see further discussion), it seems paradoxical that EM9 (DNA strand breaks repair-defective) shows up as more resistant in terms of cell growth than the parental line AA8 (repair-proficient). In our opinion, however, it is likely that in the present case is not DNA or chromosome damage the main responsible for the cytotoxic effects observed, but instead a malfunction of topo II in segregation of daughter chromatids leading to endoreduplication, and a failure to accomplish chromatin condensation should be responsible.

It is worth mentioning that the basal topo II activity appears as higher in EM9 than that found in AA8 [41], while our Western blot analysis did not show any difference in the relative amount of the nuclear enzyme.

We have found a dose-dependent induction of DNA damage by ICRF-193, as shown by Comet assay in both cell lines AA8 and EM9, while bufalin showed as completely unable to increase the values of tail moments. As to the possible differences between both cell lines in their response to the bisdioxopiperazine, in good agreement with its DNA repair-defective phenotype, the mutant EM9 cells appear as more sensitive to ICRF-193 than the repair-proficient AA8 cell line.

In general in our opinion these features deserve further discussion. While the lack of DNA damage observed after bufalin treatment can be attributable to an absence of any direct interaction of this compound with topo II, as observed by us in vitro, given the known molecular mechanism of bisdioxopiperazines such as ICRF-193 on topo II catalytic cycle, it seems rather striking its ability to result in DNA breaks. As mentioned above, ICRF-193 acts by inhibiting the intrinsic ATPase activity of topo II, stabilizing the enzyme in its closed clamp-form and sequestering it from turnover [42]. What does not seem apparent is why this anomalous blocked closed clamp form of the enzyme that "embraces" DNA might result in breakage of the molecule. A likely explanation should be that the DNA repair machinery might identify such a structure as a bulky aduct and endonucleolytic attack

should proceed, maybe eventually leading to the triggering of apoptosis via p53 when the repair capacity is overcome.

In good agreement with our observations on DNA damage, ICRF-193 was shown as a clastogenic agent working on a dose-dependent fashion, while bufalin treatment did not result in any increased yield of chromosome damage. Ikushima et al. [43] 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. While the induction of chromosome damage by the bisdioxopiperazines was found in both AA8 and EM9 cell lines, this latter showed up as more sensitive, as is to be expected from its defective DNA strand break repair phenotype [44].

Acknowledgements

The authors are greatly indebted to M.A. Ledesma for her excellent technical assistance. We are also grateful to A. Carrillo for his invaluable help with the statistical analyses. This work has been partly funded by Grants from Spanish Ministry of Science and Technology (DGI; SAF-2000-0167), and Junta de Andalucía (CVI 120), Spain.

References

[1] J.C. Wang, DNA topoisomerases, Annu. Rev. Biochem. 65 (1996) 635–692.
[2] M.D. Pommier, DNA topoisomerase II inhibitors, in: A.T. Beverly (Ed.), Cancer Therapeutics: Experimental and Clinical Agents, Humana Press, Totowa, NJ, Vol. I, 1997, pp. 153–174.
[3] M. Gupta, A. Fujimori, Y. Pommier, Eukaryotic DNA topoisomerases I, Biochim. Biophys. Acta 1262 (1995) 1–14.
[4] T. Hsieh, Mechanistic aspects of type-II DNA topoisomerases, DNA Topology and Its Biological Effects, Cold Spring Harbor Laboratory Press, 1990, pp. 243–263.
[5] L.F. Liu, DNA topoisomerase poisons as antitumor drugs, Annu. Rev. Biochem. 58 (1989) 351–375.
[6] A.H. Corbett, N. Osheroff, When good enzymes go bad: conversion of topoisomerase II to a cellular toxin by antineoplastic drugs, Chem. Res. Toxicol. 6 (1993) 585–597.
[7] L. Liu, DNA Topoisomerases: Topoisomerase-Targeted Drugs, Academic Press, New York, Vol. 29, 1994.
[8] S.J. Froelich-Ammon, N. Osheroff, Topoisomerase poisons: harnessing the dark side of enzyme mechanism, J. Biol. Chem. 270 (1995) 21429–21432.
[9] T. Andoh, R. Ishida, Catalytic inhibitors of DNA topoisomerase II, Biochim. Biophys. Acta 1400 (1998) 155–171.
[10] C.P. Belani, L.A. Doyle, J. Aisner, Etoposide: current status and future perspectives in the management of malignant neoplasms, Cancer Chemother. Pharmacol. 34 (1994) S118–126.
[11] Y. Pommier, M.R. Fesen, F. Goldwasser, Cancer Chemotherapy and Biotherapy: Principles and Practice, in: B.A. Chabner, D.L. Longo (Eds.), 2nd Edition, Lippincott-Raven, Philadelphia, PA, 1996, pp. 435–461.
[12] M.M. Heck, W.C. Earnshaw, Topoisomerase II: a specific marker or cell proliferation, J. Cell Biol. 103 (1986) 2569–2581.
[13] C.S. Downes, D.J. Clarke, A.M. Mullinger, J.F. Giménez-Abián, A.R. Creighton, R.T. Johnson, A topoisomerase II-dependent G_2 cycle checkpoint in mammalian cells, Nature (London) 372 (1994) 467–470.
[14] J.F. Giménez-Abián, D.J. Clarke, A.M. Mullinger, C.S. Downes, R.T. Johnson, A postprophase topoisomerase II-dependent chromatid core separation step in the formation of metaphase chromosomes, J. Cell Biol. 131 (1995) 7–17.
[15] W.T. Beck, M.K. Danks, Multidrug resistance associated with alterations in topoisomerase II, in: M. Potmesil, K. Kohn (Eds.), DNA Topoisomerases in Cancer. Oxford University Press, New York, 1991, pp. 260–275.
[16] P.B. Jensen, M. Sehested, DNA topoisomerase II rescue by catalytic inhibitors, Biochem. Pharmacol. 54 (1997) 755–759.
[17] F. Cortés, J. Piñero, F. Palitti, Cytogenetic effects of inhibition of topoisomerase I or II activities in the CHO mutant EM9 and its parental line AA8, Mutat. Res. 288 (1993) 281–289.
[18] P. Skehan, P. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M. R Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, J. Natl. Cancer Inst. 82 (1990) 1113–1118.
[19] Y.P. Keepers, P.E. Pizao, G.J. Peters, J. Van Ark Otte, B. Winograd, H.M. Pinedo, Comparison of the sulforhodamine B protein and the tetrazolium (MTT) assays for in vitro chemosensitivity testing, Eur. J. Cancer 27 (1991) 897–900.
[20] M.W. Heartlein, H. Tsuji, S.A. Latt, 5-Bromo-deoxyuridine-dependent increase in sister chromatid exchange formation in Bloom's syndrome is associated with reduction in topoisomerase II activity, Exp. Cell Res. 169 (1987) 245–254.
[21] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[22] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantification of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–191.
[23] D.W. Fairbain, P.L. Olive, K.L. O'Neill, The comet assay: a comprehensive review, Mutat. Res. 339 (1995) 37–59.
[24] P.L. Olive, J.P. Banath, R.E. Durand, Detection of etoposide resistance by measuring DNA damage in individual Chinese hamster cells, J. Natl. Cancer Inst. 82 (1990) 779–783.

[25] M. Chen, W.T. Beck, Teniposide-resistant CEM cells, which express mutant DNA topoisomerase II, when treated with non-complex-stabilizing inhibitors of the enzyme, display no cross-resistance and reveal aberrant functions of the mutant enzyme, Cancer Res. 53 (1993) 5946–5953.

[26] P.B. Jensen, B.S. SØrensen, M. Sehested, E.J.F. Dement, E. Kjeldsen, E. Friche, H.H. Hansen, Different modes of anthracycline interaction with topoisomerase II, Biochem. Pharmacol. 45 (1993) 2025–2035.

[27] P.B. Jensen, P.S. Jensen, E.J.F. Dement, E. Friche, B.S. SØrensen, M. Sehested, K. Wassermann, L. Vindeloev, O. Westergaard, H.H. Hansen, Antagonistic effect of aclarubicin on daunorubicin-induced cytotoxicity in human small cell lung cancer cells: relationship to DNA integrity and topoisomerase II, Cancer Res. 51 (1991) 5093–5099.

[28] R. Ishida, T. Miki, T. Narita, R. Yui, S. Sato, K.R. Utsumi, K. Tanabe, T. Andoh, Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivates: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors, Cancer Res. 51 (1991) 4909–4916.

[29] Y. Ishimi, R. Ishida, T. Andoh, Synthesis of Simian virus 40 C-family catenated dimers in vivo in the presence of ICRF-193, J. Mol. Biol. 247 (1995) 835–839.

[30] J. Roca, The mechanism of DNA topoisomerases, TIBS 20 (1995) 156–160.

[31] S. Hashimoto, Y. Jing, N. Kawazoe, Y. Masuda, S. Nakajo, T. Yoshida, Y. Kuroiwa, K. Nakaya, Bufalin reduces the level of topoisomerase II in human leukemia cells and affects the cytotoxicity of anticancer drugs, Leuk. Res. 21 (1997) 875–883.

[32] L. Zhang, K. Nakaya, T. Yoshida, Y. Kuroiwa, Induction by bufalin of differentiation of human leukemia cells HL60, U937 and ML1toward macrophage/monocyte-like cells and its potent synergistic effect on the differentiation of human leukemia cells in combination with other inducers, Cancer Res. 52 (1992) 4634–4641.

[33] S. Numazawa, M. Shinoki, H. Ito, T. Yoshida, Y. Kuroiwa, Involvement of Na+, K(+)−ATPase inhibition in K562 cell differentiation induced by bufalin, J. Cell Physiol. 160 (1994) 113–120.

[34] T. Yoshida, The presence of a common antigen between human gastric cancer cells and OK-432, Biotherapy 7 (1993) 1682–1688.

[35] D.Y. Lee, M. Yasuda, T. Yamamoto, T. Yoshida, Y. Kuroiwa, Bufalin inhibits endothelial cell proliferation and angiogenesis in vitro, Life Sci. 60 (1997) 127–134.

[36] Y. Jing, M. Watabe, S. Hashimoto, S. Nakajo, K. Nakaya, Cell cycle arrest and protein kinase modulating effect of bufalin on human leukemia ML1 cells, Anticancer Res. 14 (1994) 1193–1198.

[37] Y. Masuda, N. Kawazoe, S. Nakajo, T. Yoshida, Y. Kuroiwa, K. Nakaya, Bufalin induces apoptosis and influences the expression of apoptosis-related genes in human leukemia cells, Leuk. Res. 19 (1995) 549–556.

[38] M. Watabe, Y. Masuda, S. Nakajo, T. Yoshida, Y. Kuroiwa, K. Nakaya, The cooperative interaction of two different signalling pathways in response to bufalin induces apoptosis in human leukaemia U937 cells, J. Biol. Chem. 271 (1996) 14067–14073.

[39] M. Watabe, S. Nakajo, T. Yoshida, Y. Kuroiwa, K. Nakaya, Treatment of U937 cells with bufalin induces the translocation of Casein Kinase 2 and modulates the activity of topoisomerase II prior to the induction of apoptosis, Cell Growth Difference 8 (1997) 871–879.

[40] N. Kawazoe, M. Watabe, Y. Masuda, S. Nakajo, K. Nakaya, Tiam 1 is involved in the regulation of bufalin-induced apoptosis in human leukemia cells, Oncogene 18 (1999) 2413–2421.

[41] N. Pastor, J. Piñero, T. Ortiz, J.C. Mateos, M. De Miguel, F. Cortés, Topoisomerase activities and levels in irradiated Chinese hamster AA8 cells and in its radiosensitive mutant EM9, Int. J. Radiat. Biol. 75 (1999) 1035–1042.

[42] J. Roca, R. Ishida, J.M. Berger, T. Andoh, J.C. Wang, Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp, Proc. Natl. Acad. Sci. USA 91 (1994) 1781–1785.

[43] T. Ikushima, Y. Shima, Y. Ishii, Effects of an inhibitor of topoisomerase II, ICRF-193 on the formation of ultraviolet-induced chromosomal aberrations, Mutat. Res. 404 (1998) 35–38.

[44] L.H. Thompson, K.W. Brookman, L.E. Dillehay, A.V. Carrano, J.A. Mazrimas, C.L. Mooney, J.L. Minkler, A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange, Mutat. Res. 95 (1982) 427–440.