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Bufalin influences the repair of X-ray-induced DNA breaks in Chinese hamster cells

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Abstract

The bufadienolide bufalin, a component of the Chinese medicine *chan'su*, has been reported to selectively inhibit the growth of various lines of human cancer cells, due at least in part to its specific effect on topoisomerase (topo) II. We have treated Chinese hamster ovary (CHO) cells with doses of bufalin that result in a dramatic reduction in both the level and catalytic activity of topo II without any concomitant induction of DNA damage, as assessed by the comet assay. When cells were pre-treated with bufalin and then irradiated with X-rays, a follow-up study revealed that the kinetics of DNA repair was clearly affected, with a general delay in the restoration of DNA to the situation observed in non-irradiated controls. The possible involvement of topo II in radiation damage repair is discussed.

Keywords: DNA radiation damage repair; Topoisomerase II; Bufalin; Comet assay

1. Introduction

DNA topoisomerases (topos) play 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]. As to their possible role in DNA repair however, this represents still nowadays a rather controversial issue [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]. Taking into account the major roles played by topos in cell physiology as a whole, as stated above, it is no wonder that these nuclear enzymes are nowadays a major focus of research for cancer chemotherapy [5].

The 'classical' topoisomerase poisons, 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 through the 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, DNA and chromosome damage do arise, and cell death mechanisms are somehow triggered [5].

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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 of cytotoxic drugs of diverse nature currently available for the clinical treatment of human cancers [6–8]. Since the concentration of topo II is usually elevated in rapidly proliferating or transformed cells [9], clinically aggressive 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 [10] by preventing topo II from carrying out its required physiological functions. 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 [11,12]. These non-classical drugs have attracted clinical interest because they appear to circumvent the at-MDR phenotype [12] and may operate as antagonists to topo II poisons. They include aclarubicin, fostriecin, merbarone, suramin, quinobenoxazine, bisdioxopiperazines (ICRF-154, ICRF-193, etc.), chloroquine and novobiocin [10,13].

We have recently reported on the cytotoxic and genotoxic effects of the bisdioxopiperazine ICRF-193, long considered as a true catalytic inhibitor of topo II, and bufalin, one of the components of the bufadienolides in the traditional Chinese toad venom *chan'su* medicine able to suppress efficiently topo II activity in the cell through a mechanism as yet poorly understood [14]. While both drugs showed as cytotoxic at doses able to inhibit topo II catalytic activity, only ICRF-193 was an efficient inducer of DNA damage, in good agreement with recent reports [15–17].

In the present investigation, we have chosen bufalin, given its ability to suppress topo II activity without simultaneously causing DNA damage, to carry out an analysis of the possible modulation of DNA repair kinetics of X-irradiated CHO cells when topo II activity is depressed as a consequence of a pre-treatment with the bufadienolide agent. Our results seem to indicate that topo II has to be fully operative for DNA radiation damage repair to proceed normally.

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

The bufadienolide bufalin was obtained from Sigma (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO) (made up fresh for each experiment) and directly added to the culture medium.

2.3. Preparation of nuclear extracts

Topo II activity in nuclear extracts from AA8 and EM9 cells that had been treated with different doses of bufalin for 22 h were obtained as described by Heartlein et al. [18]. 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 × *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 × *g* at 4 °C was then collected. Total protein concentration in each extract [19] 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 1 month.

2.4. Topoisomerase II activity in nuclear extracts

Topo IIα 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.

2.5. Comet assay

AA8 and EM9 cells were treated with increasing doses of up to 5×10^{-4} M bufalin for 22 h and/or irradiated with X-rays (delivered at 1.6 cGy/s using a Philips MG103/2.25 system) and the single cell gel electrophoresis (SCGE) or comet assay was basically performed according to the original protocols of Singh et al. [20]. 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 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 [21]. 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 analyzed 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 × tail intensity or percent migrated DNA) [22].

3. Results

3.1. Loss of topoisomerase II activity after a protracted treatment with bufalin

We have recently reported on a negative effect of bufalin on cell growth [23]. Fig. 1 shows that the bufadienolide is capable of efficiently inhibiting the topo II catalytic activity of nuclear extracts from both AA8 and EM9 cells. A dramatic loss was found in the capacity to decatenate double-stranded catenated kinetoplast DNA (kDNA) as a result of bufalin treatment with a dose of 0.1 mM or higher for 22 h in both AA8 and EM9 cells (Fig. 1), in good agreement with our earlier report [23]. No apparent difference regarding the efficiency of bufalin to inhibit topo II catalytic activity was observed for both AA8 and EM9 cells (Fig. 1). On the other hand, a good correspondence between the doses of bufalin inhibiting cell growth [23] and those resulting in a clear inhibition of topo II catalytic activity was observed (not shown), supporting the hypothesis of a close relationship between both end-points [14,23–25].

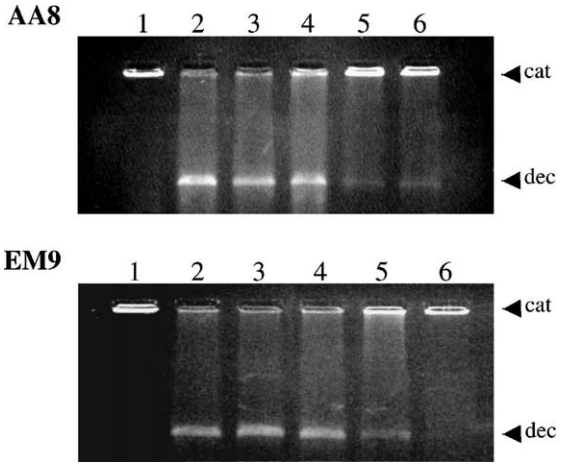

Fig. 1. Suppression of topo II catalytic activity in nuclear extracts from both AA8 and EM9 cells treated with different doses of bufalin for 22 h. Lane 1: marker catenated kinetoplast DNA (cat); lane 2: control showing efficient decatenation (dec); lane 3: 10^{-6} M bufalin; lane 4: 0.01 mM; lane 5: 0.1 mM; lane 6: 0.5 mM.

3.2. Modulation of DNA repair kinetics in X-irradiated cells pre-treated with bufalin

In order to study any possible influence of an active topo II on the final outcome of DNA repair after X-ray damage, a difficulty commonly encountered is that most of the chemicals such as etoposide so far reported to be able to reduce the enzyme presence and/or catalytic activity do induce concomitantly DNA damage. This constitutes a perturbing factor when it comes to the assessment of a possible modulation of DNA repair kinetics and makes the search for "clean" topo II suppressor drugs that by themselves do not induce DNA damage an interesting alternative.

We have recently found that in CHO cells bufalin treatment results in a loss of topo II activity, as shown above, in good agreement with that reported earlier in HL-60 human leukemia cells [14], while no chromosome nor DNA damage was detected [23].

As shown in Fig. 2, irradiation of repair-proficient parental AA8 cells with a dose of 4 Gy of X-rays was carried out, and the subsequent repair kinetics followed up by sampling at different times. Alkaline comet assay was performed and the parameter chosen to assess DNA damage was the tail moment of the comets [22]. As can be seen (Fig. 2), while the level of initial damage observed was rather high, repair seemed to proceed very efficiently in this parental cell line, in such a way that 15 min after irradiation over 60% of the cells showed tail moments within the range found in non-irradiated controls. As observed (Fig. 2), 1 h after exposure to X-rays, complete repair seems to have taken place in AA8 cells. Contrasting with this rather rapid and efficient repair, on the other hand, the repair-deficient EM9 mutant cells, when damaged with 3 Gy of X-rays (a dose comparable with 4 Gy in AA8 in terms of DNA damage), showed up as unable to carry out a rapid restoration of DNA to the situation observed in the controls (Fig. 2).

Fig. 3 shows the influence of a pre-treatment with 0.5 mM bufalin for 22 h (previously shown as efficiently reducing the topo II catalytic activity; see Fig. 1) on the repair kinetics of irradiated AA8 and EM9 cells. As can be seen, pre-treatment with bufalin, that according to earlier investigation specifically results in a loss of topo II [14] without affecting topo I nor any other protein, led to a modulation of DNA repair kinetics in both cell lines, more evident for the repair proficient AA8 cells. As compared to that shown in Fig. 2, 15 min after irradiation roughly 5% of the cells showed up as normal, contrasting with about 60% in absence of bufalin pre-treatment (Fig. 2). On the other hand, repair was not complete 3 h after irradiation in bufalin pre-treated cells, contrasting with the full repair accomplished 1 h after X-rays damage previously observed (Fig. 2).

4. Discussion

As nuclear enzymes that nick and rejoin DNA strands, topoisomerases as a whole have been considered as good candidates to play a role in the enzymatic repair processes going on in the cell after DNA damage inflicted by either physical or chemical agents, although much is left to find about the mechanism of their general action in the cell.

Contrasting with the situation in yeast, in which temperature-sensitive topo II mutants are available [26], in mammalian cells the question as to the possible involvement of topo II in DNA repair has been dealt with using alternative approaches. Most commonly, the DNA repair processes have been studied in the presence and absence of topo II inhibitors for comparison. In general, however, a clear interpreta-

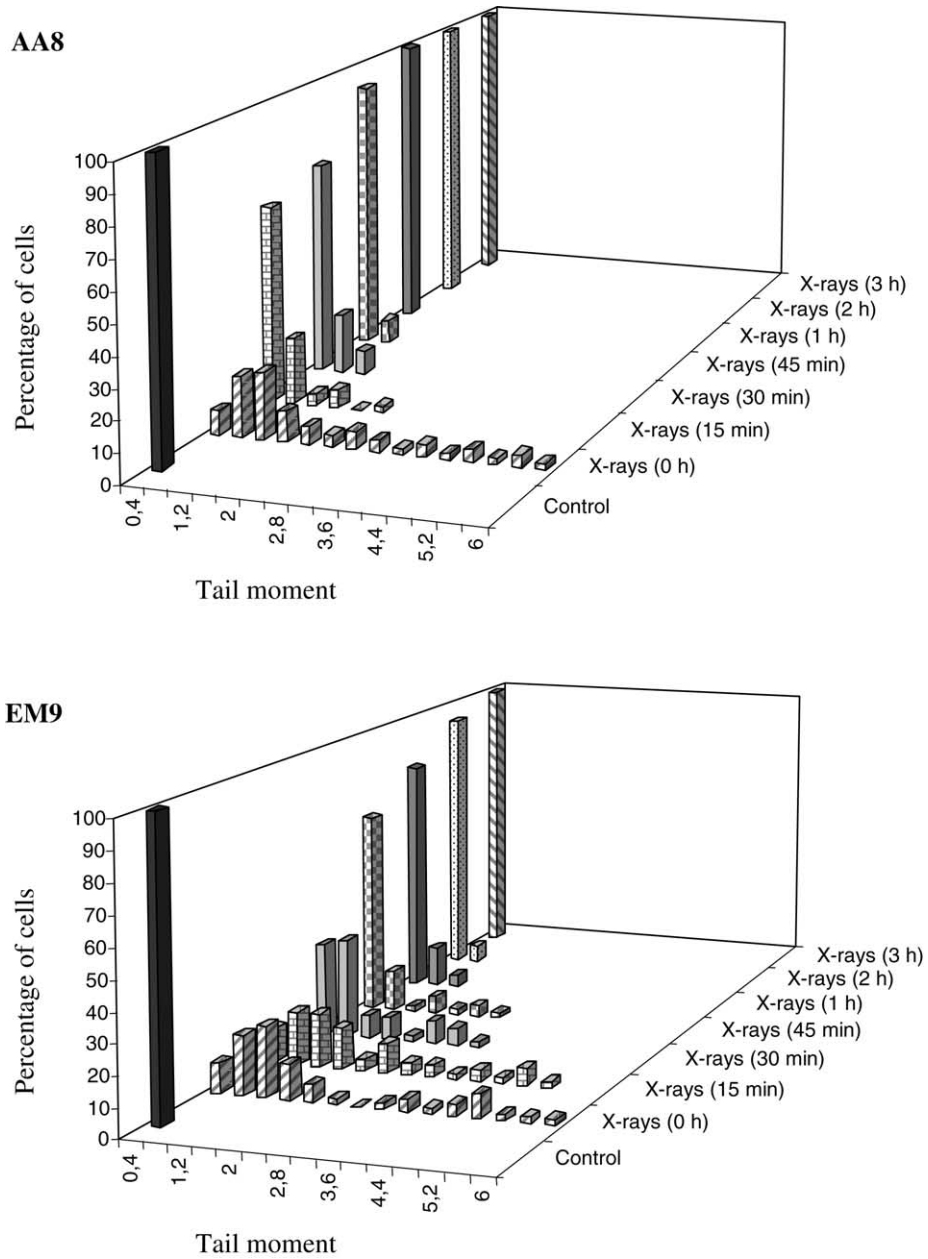

Fig. 2. Assessment of DNA radiation damage repair in AA8 and EM9 cells by the comet assay. Repair-proficient AA8 cells were given a dose of 4 Gy of X-rays, while radiosensitive EM9 cells received only 3 Gy. As can be seen, DNA repair in AA8 was apparently complete 1 h after irradiation, while the mutant cell line EM9 needed 3 h to reach full DNA repair.

tion of such studies has been difficult due to the fact that most clinically used topo II inhibitors lead to formation of DNA strand breaks, either directly or indirectly [5]. However, recent studies seem to support a role of topos in DNA repair at several different possible levels, namely: (i) the initial recognition of DNA damage; (ii) recombination; and (iii) regulation of chromatin and DNA structure [27].

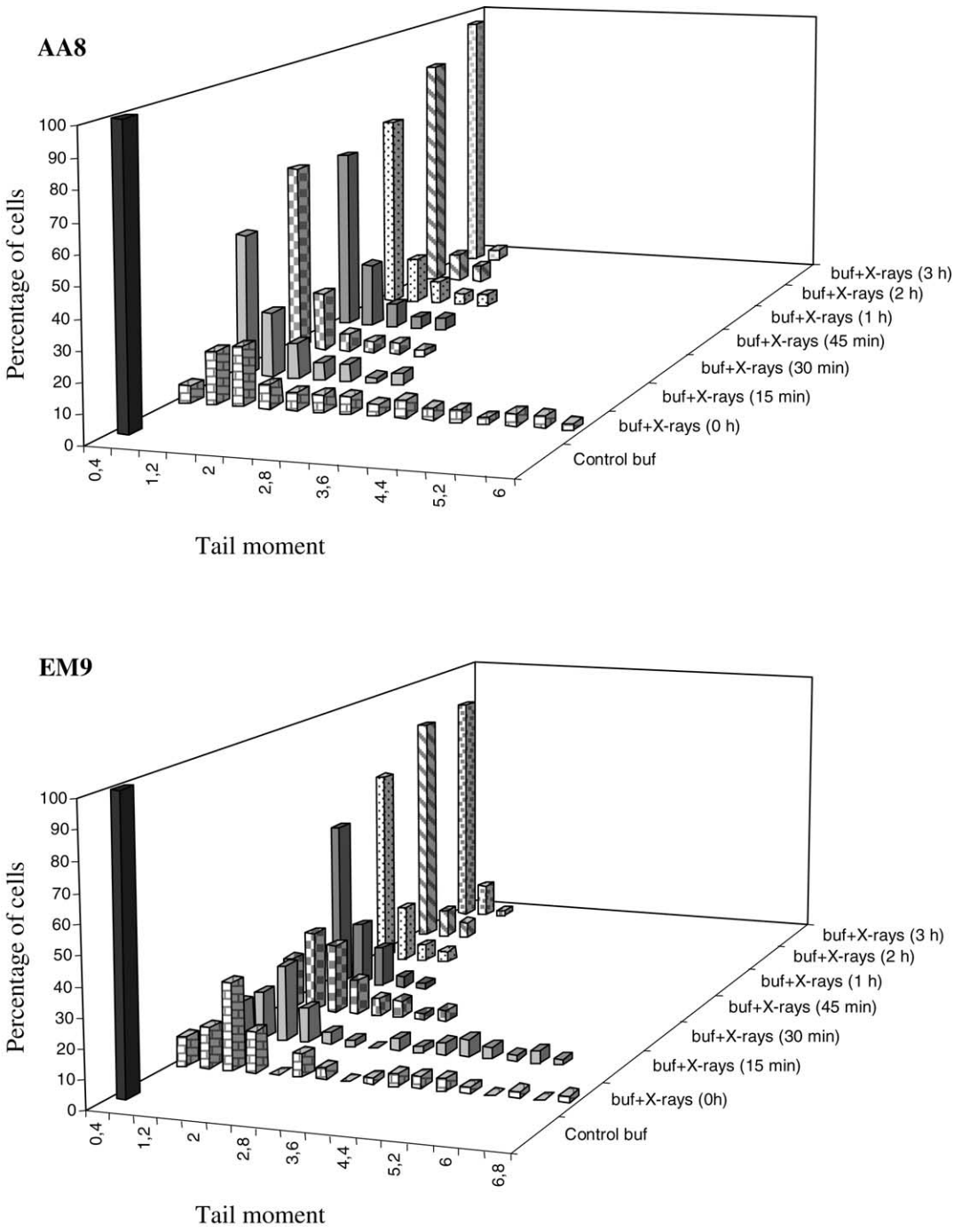

Fig. 3. Modulation of DNA repair kinetics by a pre-treatment with 0.5 mM bufalin for 22 h in irradiated AA8 and EM9 cells. Note that, while control treatment with bufalin alone didn't result in any increase in DNA damage over that found in untreated cells (black bar), repair kinetics was clearly delayed, as compared with cells irradiated in absence of any pre-treatment with the bufadienolide (see Fig. 2). Although more evident for the repair-proficient AA8 cells, the differences between just irradiated EM9 cells and those irradiated after a pre-treatment with bufalin were also statistically significant ($P < 0.05$, Student's *t*-test).

Densely packed chromatin, when damaged by UV light or high-energy radiation, is transiently loosened to render damaged sites accessible to excision repair enzymes, in particular to repair helicases and endonucleases. Topologically constrained "naked" DNA represents a bad substrate for repair enzymes, unless DNA topo IIα (or DNA topo I) would relieve the constraint thus making DNA repair easier.

According to these considerations, the action of DNA topo IIα would have a preparatory function in repair, preceding repair-specific DNA incision. In the present investigation, we have observed that X-irradiated CHO cells pre-treated with the topo II suppressor agent bufalin, a bufadienolide used in the Chinese traditional medicine that results in a loss of disponibility of the enzyme [14,23], show a dramatic delay in their DNA repair kinetics, as assessed by the comet assay. The advantage of using bufalin for our purpose of analyzing any possible involvement of topo II in radiation damage repair is that this compound has been reported to act on the cell cycle of human leukemia cells in a similar fashion to that of topoisomerase inhibitors. Indeed, the activity of topo II but apparently not that of topo I or any other enzyme such as those directly involved in DNA repair appear to be remarkably inhibited by the treatment of the cells with 10 nM bufalin in human leukemia cells, which show as highly sensitive to this compound [14], as well as in CHO cells [28], (the present report). Concerning this, however, the need of further investigation as to the possible influence, either direct or indirect, of bufalin treatment on the repair process as a whole, independently of its suppression of topo II in the cell, makes any present conclusion only provisional. This last consideration notwithstanding, an additional interesting advantage is that, contrasting with earlier reports on induction of apoptosis in human leukemia cells [29,30], we have not observed any DNA damage after a protracted treatment with bufalin [23] (the present report).

In our opinion, taking into account the previous discussion, the results reported in the present investigation seem to support that topo II has to be present and fully operative for radiation damage to be properly repaired with a normal kinetics in mammalian cells. Nevertheless, further investigation making use of agents able to suppress either topo II or topo I or both in the cell is needed for a complete understanding of their possible direct or indirect role in DNA repair. Concerning this goal, the search for topoisomerase catalytic inhibitors acting in a "clean" fashion, i.e. without any induction of DNA damage by themselves, appears as a very promising future endeavor.

A priori however, this does not seems to be an easy task, taking into account that most of the so-called topo II catalytic inhibitors, non-cleavable complex forming compounds, studied so far as to their ability to induce DNA damage have been found positive. This was the case for the bisdioxopiperazine ICRF-193 [15–17,23], and we have also observed recently that the intercalative agent aclarubicin, a topo II catalytic inhibitor that simultaneously poisons topo I, is also able to damage DNA (unpublished data).

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