Mutation Research 738–739 (2012) 45–51

Contents lists available at SciVerse ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

The DNA topoisomerase II catalytic inhibitor merbarone is genotoxic and induces endoreduplication

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ARTICLE INFO

Article history:
Received 15 March 2012
Received in revised form 3 July 2012
Accepted 19 July 2012
Available online 15 August 2012

Keywords:
Topoisomerase II
Catalytic inhibitor
Merbarone
DNA damage
Clastogens
Endoreduplication

ABSTRACT

In the last years a number of reports have shown that the so-called topoisomerase II (topo II) catalytic inhibitors are able to induce DNA and chromosome damage, an unexpected result taking into account that they do not stabilize topo II-DNA cleavable complexes, a feature of topo II poisons such as etoposide and amsacrine. Merbarone inhibits the catalytic activity of topo II by blocking DNA cleavage by the enzyme. While it was first reported that merbarone does not induce genotoxic effects in mammalian cells, this has been challenged by reports showing that the topo II inhibitor induces efficiently chromosome and DNA damage, and the question as to a possible behavior as a topo II poison has been put forward. Given these contradictory results, and the as yet incomplete knowledge of the molecular mechanism of action of merbarone, in the present study we have tried to further characterize the mechanism of action of merbarone on cell proliferation, cell cycle, as well as chromosome and DNA damage in cultured CHO cells.

Merbarone was cytotoxic as well as genotoxic, inhibited topo II catalytic activity, and induced endoreduplication. We have also shown that merbarone-induced DNA damage depends upon ongoing DNA synthesis. Supporting this, inhibition of DNA synthesis causes reduction of DNA damage and increased cell survival.

1. Introduction

Research has focused on the ubiquitous nuclear enzymes DNA topoisomerases for over 20 years, not only for their reported essential roles in gene regulation, cell cycle, mitosis and chromosome structure, but also for their importance as molecular targets of an increasing number of cancer chemotherapeutic drugs [1]. A number of reviews on the structure and functions of these enzymes in eukaryotic cells have been published [2,3].

As to their functions, DNA topoisomerases are directly involved in basic biological processes depending upon the dynamic nature and topology of the DNA molecule, such as replication, transcription, recombination, repair and segregation. While topoisomerase I (topo I) relaxes DNA supercoiling by a single-stranded DNA passage mechanism, topo II hydrolyses ATP and changes DNA topology by a double-stranded DNA passage process. This molecular mechanism of topo II allows the enzyme not only to resolve supercoiling, but is a feature that makes it unique in its capacity to decatenate intertwined double-stranded DNA molecules and resolve knots that can arise during DNA topological changes.

Drugs that affect DNA topoisomerases by interfering with their crucial function on DNA metabolism have proven to be useful for cancer treatment [1,4,5]. Merbarone (5-[N-phenylcarboxamido]-2-thiobarbituric acid; NSC 336628) has been classified as a DNA topo II catalytic inhibitor that has been proposed to act primarily by blocking topo II-mediated DNA cleavage [6]. Accordingly, it was considered as able to prevent cleavage complexes DNA-enzyme from forming (topo II suppressor) and so differs from topo II poisons such as etoposide and amsacrine [1], that stabilize covalent complexes being responsible for topo II-mediated DNA damage, thus converting this essential enzyme into a potent cellular toxin.

Supporting this, early reports concluded that merbarone does not induce genotoxic effects in mammalian cells [7–9]. Nevertheless, this concept has been challenged in the last years on the basis that merbarone has been shown to induce efficiently chromosome and DNA damage [10,11], a feature that should not be expected from its reported inhibitory mechanism on DNA cleavage [6]. Besides, the question as to a possible behavior as a topo II poison has been put forward [11], but as yet the exact molecular mechanism of action of merbarone is debated.

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http://dx.doi.org/10.1016/j.mrfmmm.2012.07.005

In the present study, we have carried out experiments making use of a number of different techniques to further characterize the mechanism of action of merbarone on cell proliferation, cell cycle, as well as chromosome and DNA damage in cultured Chinese hamster ovary AA8 cell line. The possible interaction between merbarone and the topo II poison *m*-AMSA (amsacrine) in the induction of DNA damage, and the importance of replication fork progress for the clastogenic effect of merbarone has also been investigated.

Our results seem to indicate that merbarone is cytotoxic as well as genotoxic, inhibits topo II catalytic activity, and induces endoreduplication. On the other hand, the drug is able to induce chromosome and DNA damage *per se*, while irreparable chromosome damage leading to genotoxic effect depends upon ongoing DNA synthesis. This is clearly shown as inhibition of DNA synthesis leads to a reduction of DNA damage and increased cell survival.

2. Materials and methods

2.1. Cells and culture conditions

The ovary fibroblast Chinese hamster cell line AA8 was purchased from the American Type Culture Collection (ATCC), USA. Cells were routinely maintained as monolayers in McCoy's 5A medium (BioWhittaker) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and the antibiotics penicillin (50 U/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. Sulforhodamine B (SRB) assay

Cells in exponential growth phase were harvested using trypsin-EDTA (Gibco BRL), and resuspended in medium. They were seeded at a density of 5×10^3 cells/100 μl in a 96-well microtitre plate (Nunc) and then allowed for 24 h to attach and grow. Then, they were incubated further for 48 h in the presence of merbarone. The concentration range tested (2.5–200 μM) was prepared in tissue culture medium from a 0.1 M stock solution in DMSO.

Following the recommendations of the National Cancer Institute (USA), the analysis of cytotoxic effects induced by merbarone was determined using a cell growth assay, the sulforhodamine B (SRB) assay [12,13]. 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 the proteins and fix the cells. The supernatant was then discarded, and the plates were washed five times with deionized water and air-dried. The cells were then stained with 100 μl/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). Each experiment was independently performed in triplicate.

2.3. Preparation of nuclear extracts

Topo II activity in nuclear extracts from AA8 cells was obtained as described by Heartlein et al. [14]. 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 $MgCl_2$ 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 phenylmehyl sulfonyl fluoride (PMSF), 10^{-3} M β-mercaptoethanol and 0.5×10^{-3} M dithiothreitol (DTT)). The nuclei were then pelleted 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 [15] was determined in Beckman DU-64 spectrophotometer by the Bio-Rad protein assay (Bio-Rad Laboratories) and the extracts were kept at −80 °C for no longer than a month.

2.4. Topoisomerase II activity in nuclear extracts

Topo II activity in nuclear extracts either untreated or incubated with different concentrations of merbarone was assayed using an assay kit (TopoGen, San Diego California, USA) 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 under UV illumination.

2.5. Endoreduplication assay

Actively growing cells were cultured for 3 h in the presence of different concentrations of merbarone and, after thorough washing, allowed to recover for 18 h to allow a subsequent cell cycle for the expression of endoreduplication (second cell cycle without intervening mitosis), if any [16]. Cultures that did not receive any treatment served as control. Colcemid (2×10^{-7} M) was finally added for 2.5 h for metaphase arrest. The flasks were gently shaken to dislodge the mitotic cells, which were collected by centrifugation, treated with 0.075 M KCl for 1.5 min (hypotonic treatment), fixed in methanol:acetic acid (3:1) and dropped onto clean glass microscope slides. The slides were stained with 3% Giemsa in phosphate buffer pH 6.8 and mounted in DPX. Two thousand metaphases per culture were counted and classified as normal or as made up of diplochromosomes. All the experiments were carried out in triplicate.

2.6. Comet assay

Cells were treated for 3 h with different concentrations (20, 40, 80 and 200 μM) of merbarone. Positive DNA damage controls were obtained by treating cells with 5 Gy X-rays. Irradiations were conducted using an X-ray irradiator (Philips MU 15) operated at 100 kV and a dose rate of 1 Gy/min.

The assay was basically performed according to the original protocol of Singh et al. [17]. Briefly, cells were harvested and mixed with 0.7% low-melting agarose. A total of 85 μl (approximately 10,000 cells) of the cell suspension was spread on a precoated glass slide and placed at 4 °C for 20 min. Cells were immersed in a chilled lysis solution made up of 2.5 M NaCl, 0.1 M Na_2EDTA, 10^{-2} M Tris–HCl, 1% sodium sarcosinate, pH 10, with 1% Triton X-100 and 10% DMSO added just before use. They were kept at 4 °C in the dark for at least 1 h. After lysis, the slides were placed on a horizontal gel electrophoresis unit, side by side. The tank was filled with chilled fresh alkaline solution (10^{-3} M Na_2EDTA, 0.3 M NaOH, pH 12.8) at 4 °C and, in order to detect double- and single-strand breaks as well as alkali-labile sites. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of DNA. Electrophoresis was carried out at low temperature (4 °C) for 20 min at 1.6 V cm^{-1} and 300 mA. In order to prevent additional DNA damage all the steps described above were conducted under yellow light or in the dark.

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

DNA of individual cells was viewed using an epifluorescence microscope Eclipse 50i (Nikon Corporation Instruments Company) with an excitation filter of 550 nm and barrier filter of 590 nm. Images of 50 randomly selected cells were captured by digitization from each sample. They were examined automatically using an image analysis CometScore™ v1.5. The parameter chosen to measure DNA damage was tail moment, which is an integral of the distance and amount of DNA that has migrated out of the comet "head". An increase of DNA tail moments over the control provides a measure of DNA damage.

2.7. Immunofluorescence labeling and microscopy (foci detection)

Cells were seeded on cover slips. Next day, they were treated with different concentrations of merbarone (5, 10, 15, 20 and 40 μM) for 3 h. Cells were then washed three times with PBS (10 mM sodium phosphate, pH 7.4, 140 mM NaCl, and 3 mM KCl) and then fixed with 4% paraformaldehyde at room temperature for 10 min and extensively washed in PBS. Coverslips were permeabilized with 0.25% Triton X-100 in PBS for 5 min, and then blocked in PBS with 1% Bovine Serum Albumin (BSA) and 0.1% Tween 20 for 5 min. Cells were then incubated with the primary antibody (anti-phospho-Histone H2AX (Ser 139) mouse monoclonal IgG_1, Millipore) for 1 h at room temperature at 1:1000 dilutions in blocking solution, and washed three times in blocking solution. The cells were incubated with the secondary antibody (Alexa Fuor 488 goat anti-mouse IgG (H + L), Invitrogen) for 1 h at room temperature at 1:500 dilutions in blocking solution, washed again as above and counterstained with DAPI (100 nM) for 15 min. Finally, cells were washed 3 times in PBS for 5 min and slides were mounted in anti-fade Vectashield medium.

Immunofluorescence was observed at 40-fold magnification with a Nikon microscope ECLIPSE 50i. Cells with 10 or more foci were scored as positive. For all experimental points 300 nuclei were scored. All experiments were repeated three times.

In addition, we examined the effectiveness of different concentrations of merbarone and the bisdioxopiperazine derivate ICRF-193 (BIOMOL Feinchemikalien GmbH, Hamburg, Germany) to protect from the DNA damage induced by the topo II inhibitor *m*-AMSA. In this case, cells were treated with merbarone (5 and 10 μM) or ICRF-193 (0.5 μM) for 1 h and *m*-AMSA (100 nM) was present during the last 30 min. Moreover, to shed light on the question as to whether merbarone acts as a topo II catalytic inhibitor or a poison, we have made use of the catalytic inhibitor ICRF-193 in combination with merbarone itself to assess whether the DNA damaging activity

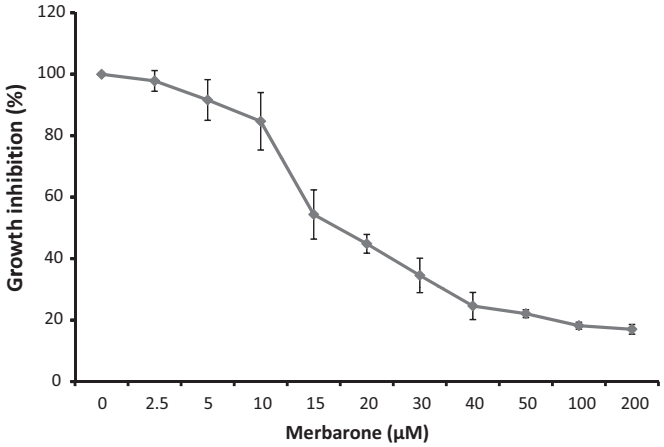

Fig. 1. Effectiveness of different concentrations of the anti-topo II merbarone to suppress cell growth in AA8 Chinese hamster ovary cells, as shown by the SRB assay. Bars indicate standard deviations of results from three independent experiments.

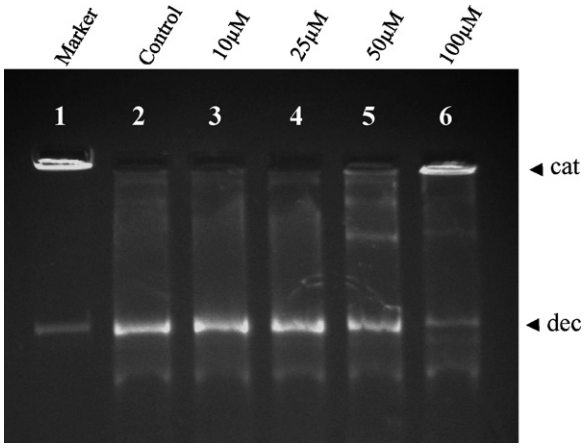

Fig. 2. Merbarone treatment and loss of topo II catalytic activity. Nuclear extracts were obtained as described in Section 2 and their ability to decatenate catenated kinetoplast DNA after incubation with different doses of merbarone ranging from 10 to 100 μM was assayed by DNA gel electrophoresis. Data show a small but consistently reduced catalytic activity of topo II as the dosage of merbarone is increased. Statistical analysis (Student's *t*-test) shows a significant difference ($P < 0.01$) for the two highest doses assayed.

of merbarone could be reversed by ICRF-193. Cells were then washed three times with PBS before being processed to visualize and quantify DSBs.

2.8. Micronucleus assay

Exponentially growing cells were incubated for 3 h in the presence of 15 μM merbarone either alone, or combinated with 3 μM of aphidicolin (APH, Sigma). This concentration of merbarone was selected according to our results from the SRB assay, since it induces a growth inhibition of roughly 50%. Treatment with APH was started 5 min before merbarone treatment. Then, cells were rinsed twice with warm medium and APH was added again for another 3 h to the culture. After treatment, cells were rinsed twice as before 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 18 h, cells were fixed in methanol-acetic acid solution (3:1). Cells were dropped onto slides and staining with Giemsa. Two thousand binucleated cells were scored for micronucleus frequency for each treatment.

2.9. Clonogenic assay

Cells were counted and seeded into six well plates (3×10^5 cells per well). Next day, they were treated for 3 h with different concentrations of merbarone either alone, or combined with 3 μM APH. Cells were then washed twice with warm medium and APH was added again for 3 h. After treatment, cells were rinsed twice, trypsinized, counted and seeded into 10 cm tissue culture dishes at appropriate densities in triplicate.

Cells were allowed to grow at 37 °C for 7 days into visible colonies. Control or treated cells were stained with Coomassie stain, and colonies containing more than 50 cells were scored as surviving cells.

2.10. Statistical analysis

For the determination of significance of the difference between the means, Student's *t*-test was used. Statistical treatment and plotting of the results were performed using the Sigma Plot and MS Excel for Windows XP software. The results come from at least two independent experiments and are presented as mean ± standard deviation of the mean. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Merbarone inhibits cell proliferation

The cytotoxicity of merbarone was determined by means of the sulforhodamine B (SRB) assay, which provides a measure of cell proliferation by quantification of the protein production of the cell as a whole [12,13]. Fig. 1 shows the results obtained for the Chinese hamster AA8 cell line when cells were treated with a range of concentrations (2.5–200 μM) of merbarone. As can be seen, the SRB assay provided results that clearly indicate a dose-dependent cytotoxic effect of merbarone on AA8 cells. A potent reduction in cell proliferation is induced at doses of merbarone higher than 10 μM, (IC_{50}: 15 μM) (Fig. 1).

3.2. In vitro inhibition of topo II catalytic activity by merbarone

We further examined the capacity of merbarone to efficiently inhibit the topo II catalytic activity in nuclear extracts from AA8 cells. The capacity of topo II to decatenate double-stranded catenated kinetoplast DNA (kDNA) was the endpoint used to assess the possible inhibition of the catalytic activity of the nuclear enzyme by merbarone. As can be seen in Fig. 2, in the absence of any drug treatment, topo II activity recovered in nuclear extracts was able to efficiently release catenated k-DNA substrate as shown by the release of closed minicircles. When the possible inhibition exerted by increasing concentrations of merbarone, ranging from10 to 250 μM, was tested, the observation was a dose-dependent inhibitory effect, as shown by a progressive increase in the amount of catenated DNA substrate remaining in the wells (Fig. 2). Doses higher than 100 μM induced a total inhibition of topo II activity (data not shown).

3.3. Induction of endoreduplication by merbarone

Once established that merbarone, at the doses tested by us, is capable of efficiently inhibit topo II catalytic activity as shown by a loss in the yield of decatenation of catenated kDNA in AA8 cells, we wanted to analyze the possible influence of this compound on normal chromosome segregation. The endpoint chosen by us to assess missegregation leading to aberrant mitosis was endoreduplication [18,19], typically visible as metaphases made up of diplochromosomes.

Treatment with different doses of merbarone took place for 3 h before allowing the cells to recover for an additional cell cycle during which endoreduplication might take place. As can be seen in Fig. 3, endoreduplication was effectively induced in a dose-dependent fashion at doses of merbarone ranging from 2.0 to 10 μM. At higher concentrations (15 μM and 20 μM) the percentage of endoreduplicated cells decreased, most likely due to a negative effect of the treatment on the rate of cell proliferation.

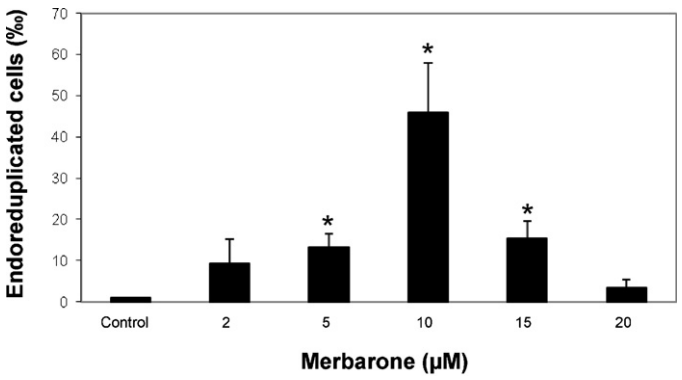

Fig. 3. Endoreduplication induced by different concentrations of merbarone. Bars represent standard deviations (S.D.) of the mean from three independent experiments (*$P < 0.01$, according to Student's *t*-test).

3.4. DNA damage induced by merbarone

The induction of DNA damage by merbarone was first investigated using 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. Following a 3 h treatment with merbarone, DNA damage was observed in cells at concentrations ranging from 20 to 200 μM (Fig. 4). It becomes apparent that merbarone treatment for 3 h results in DNA damage, as shown by clearly increased values of the Comet tail moment [20].

The DNA-damaging activity of merbarone was confirmed using the γH2AX foci detection technique, which specifically allows to assess DNA double strand breaks (DSBs). Fig. 5A shows that a 3 h-treatment of cells with various concentrations of merbarone (5–40 μM) induced a dose-dependent increase in foci formation. These findings are in line with the results obtained in the Comet assay and show that merbarone produces DNA damage *per se* in cultured mammalian cells.

It has been reported that catalytic inhibitors of topo II effectively antagonize the ability of topo II poisons to poison the enzyme [21,22]. Consequently, DNA double strand break formation does not occur if topo II is catalytically inactivated. Accordingly, we wanted to assess the effectiveness of merbarone, considered as a pure catalytic inhibitor, to reduce the level of DNA damage induced by the well known topo II poison *m*-AMSA.

Fig. 5B represents the data obtained using foci detection analysis for the quantification of DNA double-strand breaks induced by merbarone and *m*-AMSA either separately, or by a combination of merbarone plus *m*-AMSA. In this case, cells were treated with merbarone (5 and 10 μM) for 1 h and *m*-AMSA (100 nM) was present during the last 30 min. Figure clearly shows that there is not any significant increment or reduction in the level of DSBs observed in the combined treatment compared to that induced by *m*-AMSA. These results could be interpreted as merbarone and *m*-AMSA work on the same target and therefore would suggest that they both are topo poisons. In order to examine the question as to whether merbarone should be considered as a catalytic inhibitor or a poison, we carried out a series of experiments in the presence of ICRF-193, considered as a pure topo II catalytic inhibitor. Fig. 5B shows that, as expected, ICRF-193 is effective to reduce the level of DNA damage observed following treatment of AA8 cells with the topo II poison *m*-AMSA. We also assayed ICRF-193 in combination with merbarone to assess whether the damaging activity of merbarone could also be reversed by a catalytic inhibitor. In this case, we wanted to analyze the action of merbarone considered as a topo II poison. In Fig. 5B we can see that ICRF-193, instead of reducing the DNA damage induced by merbarone (as expected if merbarone was a topo II poison), increased DBSs, being these values significantly higher for the combined treatment.

3.5. Replication fork progression is required for processing of DNA damage induced by merbarone

It is known that DNA damage induced by topoisomerase poisons is related to their interaction with DNA replication fork progression [23]. Next, we wanted to address if the same occurs with merbarone. To examine this possible mechanism of poisoning of the enzyme, micronuclei formation assay was used to detect the chromosomal damage induced in cells treated during 3 h with merbarone alone or in the presence of the DNA synthesis inhibitor aphidicolin (APH). First, cell cycle progression was evaluated by classifying 1000 cells from the same slides according to the number of nuclei (binucleated (BN)/mononucleted (MN) from two independent experiments. Values expressed as percent of binucleated

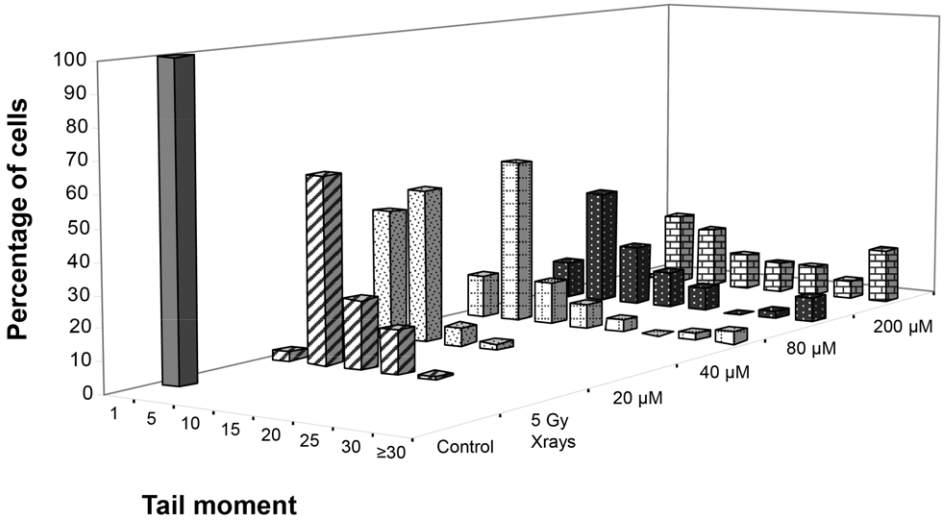

Fig. 4. Effectiveness of different doses of merbarone to induce DNA damage in AA8 cells, as shown by the Comet assay. Cells exposed to 5 Gy X-rays were used as a positive control. Merbarone seems to be very efficient in inducing DNA strand breaks. Note that merbarone at 20 μM produced values of DNA damage similar to those induced by 5 Gy of ionizing radiation. Data from three independent experiments (50 cells were measured per experimental point in each experiment). See the dose-dependent increase in tail moments, as compared to non-treated controls ($P < 0.0001$; Student's *t*-test).

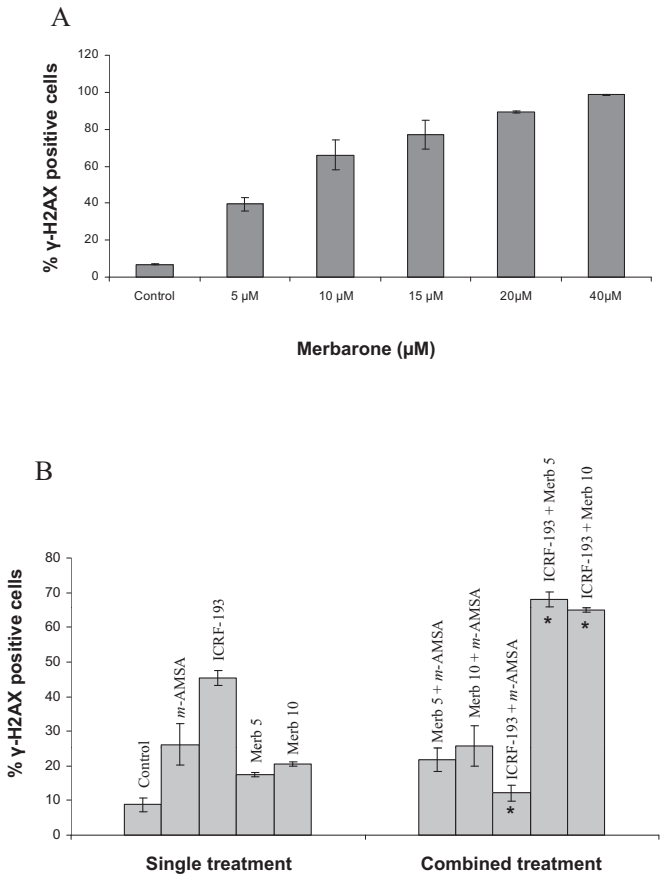

Fig. 5. Merbarone induces dose-dependent γ-H2AX foci (A) quantification of γ-H2AX positive cells (more than 10 foci) treated with different concentrations of merbarone for 3 h. Each bar represents the mean of three independent experiments ± S.D. Foci formation in merbarone-treated cells was in all the cases significant compared with non-treated cells ($P < 0.01$; Student's *t*-test). Cells were fixed and stained with anti-γ-H2AX and observed by immunofluorescent microscopy. (B) Effectiveness of merbarone to protect from DNA damage induced by the topo II poison *m*-AMSA. In this case, cells were treated with merbarone for 1 h, and *m*-AMSA was present during the last 30 min. See that any significant reduction in the level of foci in the combined treatment compared to *m*-AMSA was observed. The possible effectiveness of ICRF-193 to antagonize the DNA damage induced by *m*-AMSA or merbarone (Merb) was also analyzed as described in Section 2.7. Combined treatment (ICRF-193 + *m*-AMSA) resulted in a significant reduction in foci formation (*$P = 0.01$; Student's *t*-test) compared to *m*-AMSA alone. Combined treatment (ICRF-193 + merbarone) resulted in a significant increase in foci formation (*$P \leq 0.01$; Student's *t*-test) compared to merbarone alone.

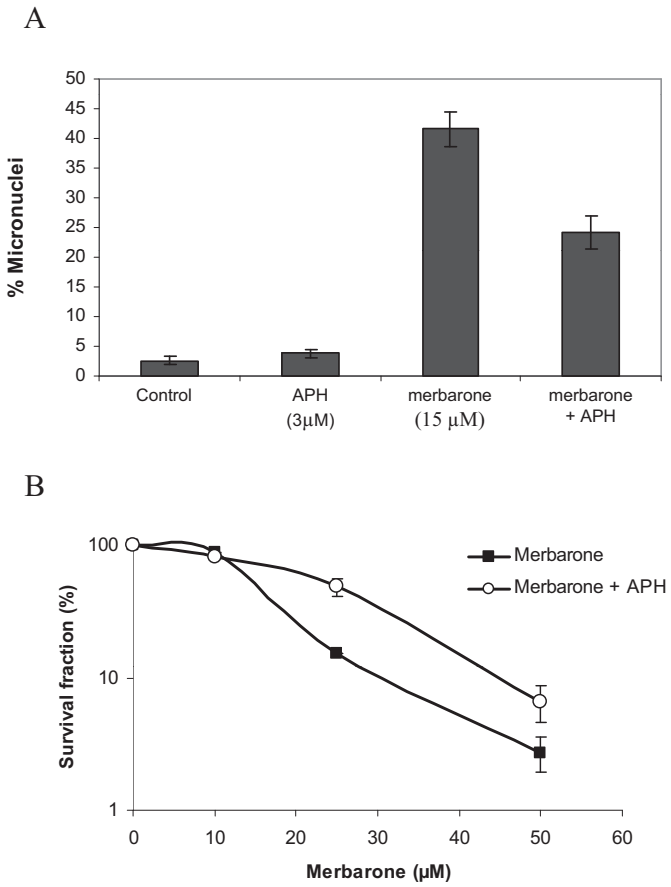

Fig. 6. (A) Frequency of micronuclei in binucleated AA8 cells treated with merbarone alone, or with merbarone plus the DNA synthesis inhibitor aphidicolin (APH). Note that the presence of APH resulted in a reduction on the frequency of micronuclei generated by merbarone. Statistical analysis (Student's *t*-test) show a significant difference ($P < 0.05$) between observed and expected data. (B) Colony-forming ability in AA8 cells treated with merbarone + APH compared to those treated with merbarone alone. After 7 days, the number of surviving colonies was counted. These experiments were performed in triplicate. Bars indicate standard deviation of the mean. Statistical analysis (Student's *t*-test) shows a significant difference ($P < 0.05$) for the two highest doses of merbarone employed.

cells (BNC) ± S.D., did not show any significant difference ($P = 0.25$, according Student's *t*-test) between the proliferation potential of cells treated with merbarone alone (BNC = 63.6 ± 4.4) and those cells treated with merbarone plus aphidicolin (BNC = 70.4 ± 6.6). As can be seen in Fig. 6A, merbarone at a dose of 15 μM was able to induce a high percentage of micronuclei as compared with non-treated control. When merbarone was administered in the presence of APH, a reduction in the number of micronuclei was observed. Similarly, cell survival of those cells treated with merbarone alone was significantly reduced compared to those obtained from cells treated with merbarone and aphidicolin simultaneously (Fig. 6B). These results seem to indicate that chromosome damage expressed as post-mitotic micronuclei produced by merbarone is a process highly influenced by the collision of DNA replication forks with the topo II enzyme-inhibitor complex. Overall, we show that DNA replication is a critical factor determining the toxicity of merbarone.

4. Discussion

In a broad sense, topo II targeting drugs are classified into two categories according to their way of interaction with the enzyme [24]. The first class is made up by the so-called topo II poisons for these agents, rather than inhibiting the overall catalytic activity of the nuclear enzyme, act by increasing the levels of topo II-mediated DNA cleavage and generate lesions that include stabilized complexes topo II-DNA and DNA strand breaks.

A second class includes chemicals able to inhibiting the enzyme catalytic activity without increasing the number of topo II covalent complexes and accordingly they are not expected to induce DNA damage.

The induction of topo II-mediated DNA damage has shown as an effective strategy for cancer chemotherapy [1,25]. Nevertheless, when it comes to topo II poisons such as etoposide and teniposide, there are important negative consequences, since it has been reported that they could lead to secondary malignancies several years after treatment, most likely due to their ability to generate translocations that lead to specific types of leukemia [26–28]. Taking into account this important drawback of topo II poisons, an interesting question is whether catalytic inhibitors might be active anticancer drugs without the undesired long-term consequences of topo II poisons.

As mentioned above, since topo II catalytic inhibitors are not expected to increase the level of topo II-DNA complexes responsible for DNA damage, they were welcome as a promising alternative to topo II poisons. In fact, early reports seemed to support that topo II catalytic inhibitors such as merbarone [7–9] and bisdioxopiperazines [29] did not stabilize cleavable complexes DNA-topo II and were ineffective as DNA damaging agents. Nevertheless, more recent investigations have shown that merbarone and the structurally related bisdioxopiperazines (ICRF-193, and ICRF-187) induced strong dose-dependent genotoxic effects in mammalian cells similar to those seen with the topo II poison etoposide. In addition, clastogenic effects could be seen *in vivo* in bone marrow erythrocytes from merbarone-treated mice [10,11,30–33]. The striking difference between the numerous negative results and the latter has not been satisfactorily explained so far. This notwithstanding, in view of the positive reports on genotoxicity and clastogenicity of the drug, the hypothesis that merbarone could behave as a topo II poison has been proposed [11] but as yet the exact mechanism of action of the drug is poorly understood.

Given the contradictory data, in an attempt to further analyze these questions, we have found that merbarone is cytotoxic to cultured AA8 Chinese hamster cells and effectively induces DNA damage. We have also shown that merbarone depends on ongoing DNA synthesis for its genotoxic effect, as shown by the observation that inhibition of DNA synthesis causes reduction of DNA damage and leads to an increase in cell survival. On the other hand, our data in relation to the ability of merbarone to attenuate cleavage enhancement induced by a topo II poison show some discrepancies with a previous report [6]. However if we have a closer look at Fig. 5B, we can observe, at least for the lower dose of merbarone assayed, a slight reduction although not statically significant of DNA damage induced by merbarone in the presence of *m*-AMSA compared to merbarone itself. Data from the figure also show that the combined treatment does not result in increased DNA damage for any of the doses used. Thus, it is likely that the apparent discordant findings with respect to merbarone/*m*-AMSA interactions are a consequence of the biological system used, concentration range evaluated, endpoint measured and perhaps other variables. These results along with the significantly higher DNA damage for the combined treatment (ICRF-193 + merbarone) (Fig. 5B), seem to point out that merbarone, although primarily a topo II catalytic inhibitor [6], can damage DNA through an as yet unknown mechanism depending upon replication.

Under natural conditions, DNA is most vulnerable during the S phase of the cell cycle. Large protein complexes (replisomes) are known to progress along DNA during its semiconservative replication. In this ongoing process, positive supercoils are produced ahead and negative supercoils behind the moving replication forks [34]. Topo II topological changes in DNA by breaking and joining double strands are necessary to allow such a replication fork progression by relieving the local torsional stress [35]. Interestingly, merbarone has been shown to delay cell cycle progression during S phase [8]. It has been proposed that, as a consequence of topo II catalytic inhibition at this stage, collapse of stalled replication forks could result in DNA strand breaks [8,36,37] which can compromise genome integrity if not properly processed by cellular DNA repair mechanisms. A similar hypothesis has been put forward for the possible consequences of topo II inhibition during transcription, *i.e.* collision of the transcription-driven RNA polymerases on template DNA could also lead to DNA damage [38].

As to the cell cycle effects, as mentioned above, it has been reported that merbarone delays progression during S-phase and causes cells to arrest in G2, delaying entry into mitosis [8,39]. It has been reported that merbarone is aneugenic as well as clastogenic [33]. In good agreement with our earlier reports on the effectiveness of different topo II inhibitors to induce endoreduplication [16,40], in the present investigation we have observed that merbarone also inhibits topo II catalytic activity in cultured Chinese hamster ovary AA8 cell line and induces endoreduplication under conditions where also DNA damage is induced.

The effectiveness of topo II inhibitors to induce endoreduplication and other forms of polyploidy in mammalian cells has been proposed to be related to the impairment of the unique role of the enzyme for a correct DNA segregation after replication of sister chromatids, essential for their separation during anaphase [3]. In fact, depletion of topo IIα by siRNA has been shown to be associated with severely reduced decatenation activity [41].

Our results on endoreduplication induced by merbarone seem to support this deleterious effect of the drug on mitosis. After prolonged inhibition of topo II decatenation, cells with catenated chromosomes cannot progress through anaphase and are either bound to die by apoptosis or re-enter into replication. G2 checkpoints protect genome integrity by delaying cellular progress in the event of DNA damage or other chromosomal alterations [42]. Checkpoint sensors inspect DNA throughout replication and mitotic division and signal to the downstream cascade of events, thereby strictly controlling the timing and sequence of cell cycle [43]. Suppression or mutated expression of DNA damage checkpoint or decatenation checkpoint genes can result in genetic instability [44], leading to polyploidy, aneuploidy, and tumorigenesis.

As to the gene products involved in the G2 decatenation checkpoint, it has been reported that it depends on BRCA1 [45] as well as on ATR and others [46]. More recently, it has been demonstrated that Rad9A is also required for G2 decatenation checkpoint and to prevent endoreduplication as a consequence of topo II catalytic inhibition [47].

In our opinion, given the importance of the decatenation process for genetic stability and the possible consequences of errors as responsible for tumor initiation and progression, further investigation is needed for a complete picture of the different genes and factors involved.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

This work has been partly funded by Grants from Spanish Ministry of Education and Science (BFU2007-61301), and Junta de Andalucía (BIO-120), Spain.

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