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Merbarone Induces Activation of Caspase-Activated DNase and Excision of Chromosomal DNA Loops from the Nuclear Matrix[§]

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Non-standard Abbreviations: MARS, matrix attached regions; CAD, caspase-activated DNase; ICAD, inhibitor of CAD; DFF, DNA fragmentation factor; AIF, apoptosis inducing factor; PCR, polymerase chain reaction; IDV, integrated density value, PTPC mitochondrial permeability transition pore complex.

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ABSTRACT

Studies were carried out to address possible cellular mechanisms by which merbarone, a catalytic inhibitor of DNA topoisomerase II, can block tumor cell growth without inducing extensive DNA cleavage. Merbarone induced the release of high molecular weight DNA fragments from the nuclear matrix of HL-60 leukemia cells, which preceded the internucleosomal-size DNA fragmentation characteristic of late stage apoptosis. The chromatin fragments were enriched in a matrix attachment region (MAR) sequence compared to a non-MAR sequence, and were similar in size to DNA loops extracted from nuclear matrices. However, merbarone did not directly induce the excision of high molecular weight DNA fragments from the nuclear matrix by promoting topoisomerase II catalyzed DNA cleavage, since the drug inhibited topoisomerase II-mediated cleavage in isolated nuclear matrix preparations. Instead merbarone induced rapid activation of the mitochondrial apoptosis pathway, which included the following temporal sequence of events: dissipation of the mitochondrial transmembrane potential within 30 min, release of mitochondrial cytochrome c, and activation of caspase-activated DNase by cleavage of its inhibitor ICAD. The excision of high molecular weight DNA was inhibited at least 80 % in merbarone treated cells preincubated with the pan-caspase inhibitor, z-VAD-fmk and in caspase-resistant Jurkat cells (ICAD/DM) that express a mutant form of ICAD. These results provide evidence that merbarone can induce rapid disorganization of DNA in tumor cells having a functional mitochondrial apoptosis pathway without inducing extensive DNA cleavage.

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DNA topoisomerase II-active agents are among the most widely used cancer chemotherapeutic drugs. Most of these drugs are referred to as "cleavable complex"-forming agents, which includes the epipodophyllotoxins (VP-16, VM-26), anthracyclines (doxorubicin, daunorubicin), anthracenediones (mitoxantrone), and the aminoacridines (*m*-AMSA). Cleavable complex-forming topoisomerase II- active drugs stabilize a covalent enzyme-DNA ternary complex and thereby interfere with the ability of the enzyme to religate DNA (Liu et al., 1983; Osheroff, 1989). VM-26 and *m*-AMSA induce the release of newly replicated DNA from the nuclear matrix of CEM leukemia cells compared to cleavage of nonmatrix DNA (Fernandes et al., 1988; Lambert and Fernandes, 2000). The resultant blockade in the movement of DNA replication forks and transcriptional complexes are critical for the development of the cytotoxic effects of these drugs (Catapano et al., 1997; D'Arpa et al., 1990).

A newer class of topoisomerase II-active agents, termed "catalytic inhibitors", is currently under clinical development. This chemically diverse group of compounds includes merbarone, aclarubicin, fostriecin, the bisdioxopiperazines, etc. Catalytic inhibitors do not stabilize topoisomerase II cleavable complexes and are much less potent inducers of DNA strand breaks compared to the cleavable complex drugs (Drake et al., 1989; Ishida et al., 1991). In fact, merbarone is thought to inhibit topoisomerase II by blocking enzyme-mediated DNA cleavage (Fortune and Osheroff, 1998). However, it is unclear whether cytotoxicity induced by catalytic inhibitors results solely from inhibition of topoisomerase II catalytic activity. It has been reported that merbarone and bisdioxopiperazines induce various chromosomal aberrations (Stanulla et al., 1997; Wang and Eastmond, 2002). This indicates that the catalytic inhibitors of topoisomerase II have the potential to induce higher-order chromosomal fragmentation.

In eukaryotic cells chromosomal DNA is organized into a hierarchy of supercoiled structures that result in at least a 10,000-fold compaction of the DNA within the nucleus (Pienta and Coffey, 1984). The most basic level of organization consists of the folding of the DNA into 50-100 kb loops, which are further wound into nucleosomes (Pienta and Coffey, 1984; Vogelstein et al., 1980). Microscopic and biochemical studies have shown that the DNA loops are attached at their bases to the nuclear matrix (Gasser and Laemmli, 1986; Vogelstein et al., 1980). DNA sequence motifs, termed matrix attachment

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regions or MARS, mediate the binding of the chromatin loops to the nuclear matrix (Cockerill and Garrard, 1986). The MARS also contain one or more matches to the topoisomerase II consensus sequence (Adachi et al., 1989; Cockerill and Garrard, 1986; Spitzner and Muller, 1988). DNA topoisomerase IIα is a major protein of the isolated nuclear matrix (Danks et al., 1994). It is thought to act at the MARS to relieve the positive supercoiling that accumulates ahead of the moving DNA replication forks and transcription complexes (Wu et al., 1988). An initial DNA event involved in apoptosis, which precedes the formation of oligonucleosome ladders, is the cleavage of DNA into 50-300 kb fragments (Lagarkova et al., 1995). These fragments represent single DNA loops and higher order rosettes of 2-6 loops (Alison and Sarraf, 1995; Pienta and Coffey, 1984). The results reported herein show that incubation of HL-60 leukemia cells with merbarone leads to the release high molecular weight DNA fragments from the nuclear matrix prior to the formation of apoptotic DNA ladders. This suggests that the chromosomal fragmentation induced by merbarone is an early event in drug-induced apoptosis. However, it is unlikely that merbarone inhibition of topoisomerase II directly releases the DNA loops from the nuclear matrix, since merbarone inhibits topoisomerase II activity by blocking enzyme-mediated DNA cleavage and does not stabilize topoisomerase II cleavable complexes (Fortune and Osheroff, 1998).

A clue regarding a possible mechanism by which merbarone can induce release of DNA loops comes from studies which show that topoisomerase II binds to and enhances the activity of caspase-activated DNase (CAD or DFF40) (Durrieu et al., 2000; Widlak et al., 2000). This endonuclease is critical for oligonucleosomal DNA degradation and late stage chromatin condensation (Enari et al., 1998; Liu et al., 1997). Upon caspase activation and subsequent cleavage by caspase-3 of the CAD inhibitor (ICAD or DFF45), ICAD dissociates from CAD. This releases active CAD, which can then catalyze chromatin digestion in the internucleosomal linker DNA, leading to formation of mono- and oligo-nucleosomal DNA fragments of ~200 bp. Of particular relevance, recent data also supports the role of CAD in the cleavage of DNA into 50-300 kb fragments during the early stages of apoptosis (Sakahira et al., 1999; Widlak, 2000). Our results are consistent with the hypothesis that merbarone induces release of

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cytochrome c from mitochondria and proteolysis of ICAD by caspase-3. The free CAD then binds to topoisomerase II at the bases of DNA loops on the nuclear matrix, where it is further activated and catalyzes the excision of high molecular weight DNA from the nuclear matrix.

MATERIALS AND METHODS

Materials - Human HL-60 leukemia cells were obtained from Dr. Yi-Te Hsu of the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC. Jurkat cell transformants expressing either a double mutated ICAD (ICAD/DM), as described by Sakahira *et al.* (Sakahira et al., 1998), or the empty retroviral vector pBabe were kindly provided by Dr. Damu Tang of the Father Sean O'Sullivan Research Institute, St. Joseph's Hospital, (Hamilton, Ontario, Canada) (Wu et al., 2002). All tissue culture reagents were obtained from Invitrogen (Carlsbad, CA). [4-^{14}C]-thymidine (specific radioactivity of 0.05 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA. VM-26 was a gift of Bristol-Myers Squibb (Princeton, NJ) and merbarone was purchased from Calbiochem (La Jolla, CA). Merbarone was prepared as a 100X stock solution in 0.1 N NaOH and VM-26 was prepared as a 1000X stock solution in DMSO/ethanol (1:1), respectively. The pan-caspase inhibitor, z-VAD-fmk was purchased from R&D Systems, Inc. (Minneapolis, MN). Antibodies for immunoblotting were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PCR primers were synthesized by Operon Technologies (Alameda, CA). Immobilin-P PVDF transfer membranes and all other chemicals and supplies were obtained from Fisher Scientific (Suwanee, GA).

Measurement of Cell Growth, Caspase Inhibition, and Apoptosis - HL-60 and Jurkat cells were grown at 37 ^{o}C under 5% CO_2-95% humidified air in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (10,000 units/L), and streptomycin (10 mg/L). The tumor cells were counted using a Coulter model Z_f counter. Exponentially growing HL-60 or Jurkat cells were resuspended in a fresh medium plus fetal bovine serum at a density of 2 or 3 x 10^5cells/ml, respectively. After 24 h, the cells were incubated with 100 μM merbarone for 5 h in all of the experiments unless otherwise indicated. In the caspase inhibition assay, the cells were pretreated with 20 μM z-VAD-fmk for 30 min and then

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subjected to the drug treatment as above in the continuous presence of z-VAD-fmk for various time periods. The loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) following drug treatment was measured by flow cytometry using the potential-sensitive fluorescent dye, JC-1, according to the manufacturer's instructions (BioVision, Mountain View, CA). The cationic dye accumulates in the mitochondria of non-apoptotic cells and forms red-fluorescent aggregates, while green monomers are distributed throughout the cell when there is loss of $\Delta\Psi_m$. Thus, the loss of $\Delta\Psi_m$ is indicated by the decrease in the ratio of the red-fluorescent aggregates to the green-fluorescent monomers.

Analysis of DNA Fragmentation - DNA fragmentation was analyzed by a modified gel electrophoresis procedure as described previously by Barry and Eastman (1993). The method involves extraction of the DNA with RNase A and SDS/proteinase K in the gel during electrophoresis. This avoids fragmentation of the DNA during extraction, and allows analysis of drug-induced higher- order DNA fragmentation. Electrophoresis was performed at 45 V for 16 h at room temperature and the DNA was stained for 1 h with ethidium bromide, followed by a 3 h washing in distilled water. DNA fragmentation was visualized under UV light and quantitated by counting the total number of pixels in each band (integrated density value, IDV) with a ChemiImager digital imaging system (Alpha Innotech Corp., San Leandro, CA).

Release of DNA Loops From the Nuclear Matrix - Briefly, exponentially growing cells (4×10^6 cells) were encapsulated in a 2 % low-melting point agarose gel, and extracted once with 1.5 M NaCl in TE buffer (20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF) for 1 h at 4 ^oC. High–salt extraction allows the removal of the histone and other soluble proteins from the nuclear matrix, leaving the integral matrix proteins, such as topoisomerase II, and the matrix attached chromosomal loops as a residual structure. High-salt extracted nuclei were then washed three times in TE buffer with 1 mM PMSF for 30 min at 4 ^oC, followed by the incubation in DNA cleavage buffer (TE buffer with 1 mM PMSF and 5 mM $MgCl_2$) for 20 min at 37 ^oC as previously described (Solovyan et al., 2002). To determine the effects of drugs on topoisomerase-II mediated excision of DNA loops, either merbarone (100 μM) or VM-26 (20 μM) were added to the high-salt extracted nuclei in DNA cleavage buffer and incubated as above. After incubation,

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the agarose gels were melted at 85 ^{o}C for 1 min and the DNA was subjected to fragmentation analysis as described above, except that the gel electrophoresis was performed without proteinase K and either in the absence or presence of SDS.

PCR Analysis of MAR and non-MAR Sequences - Following radiolabeling with 2 mM [^{14}C]thymidine for 24 h at 37 ^{o}C, HL-60 cells were incubated with and without 100 μM merbarone for 5 h and the DNA was subjected to agarose gel electrophoresis as described above. The bands from the agarose gel containing either high molecular weight DNA released from the nuclear matrix or bulk DNA that did not migrate into the separating gel were excised from the gel and the DNA was extracted for 24 h at 37 ^{o}C into a 3 volumes of TE buffer. MARS within the human c-*myc* and β-globin genes were identified using the S/MARt database on scaffold/matrix attachment regions (Liebich et al., 2002) and as described by Chou *et al*. (1990). PCR amplification of was performed targeting a 203 bp sequence within a strong MAR in exon 3 of the c-*myc* gene using primer pairs (5'-ACCATCCCTGTTTGTTTTCATC-3' and 5'-CTACCTCTCACCTTCTCACC-3'). MARS contain DNA that comprises part of DNA loops that are stably attached to the nuclear matrix. MARS also contains DNA replication origins and topoisomerase II binding sites (Adachi et al., 1989; Cockerill and Garrard, 1986; Spitzner and Muller, 1988). It is more difficult to identify non-MAR DNA sequences, since in transcriptionally active genes there are numerous, low affinity sites that bind transiently to the nuclear matrix when the gene is being transcribed (Gasser and Laemmli, 1986; Vogelstein et al., 1980). Thus, it is possible, using the S/MARt database alone, to mistakenly identify a DNA sequence motif as a MAR. To minimize this possibility, we chose to analyze a non-MAR DNA sequence motif in intron 1 of the β-globin gene, which is also transcriptionally inactive in HL-60 cells and does not contain a topoisomerase II consensus sequence. A 296 bp sequence approximately 3.5 kb upstream from the MAR in intron 1 of the human β-globin gene was amplified by PCR using primer pairs (5'-CACTAGCAACCTAAACAGAC-3'and 5'-CCCCAAAGGAC TCAAAGAAC-3').

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Equal amounts of [^{14}C]DNA containing either high molecular weight DNA that was released from the nuclear matrix (50-300 kb) or bulk DNA that did not migrate into the separating gel were used for PCR using the HotStarTaq DNA polymerase kit (QIAGEN, Inc., Valencia, CA). The HotStarTaq DNA polymerase was activated by a 15 min incubation at 95 ^{0}C in the thermal cycler. For the c-*myc* MAR this was followed by template denaturation for 40 s at 96 ^{0}C, primer-template annealing for 1 min at 53 ^{0}C, and then primer extension for 40 s at 72 ^{0}C. After 32 cycles the extension reaction was continued for an additional 7 min at 72 ^{0}C. Amplification of the non-MAR DNA sequence in the β-globin gene was carried out similarly with the exception that the primer-template annealing cycle was for 1 min at 55 ^{0}C and primer extension was for 30 s at 72 ^{0}C.

Western Blot Analysis – To measure release of AIF or cytochrome c from mitochondria, HL-60 cells were incubated for 0 to 6 h with merbarone. The cells were lysed by homogenization in ice-cold 10 mM Hepes/KOH buffer, pH 7.5 containing 200 mM manitol, 68 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and proteinase inhibitor solution (1 mM PMSF, 1 mM benzamidine, soybean trypsin inhibitor (10 μg/ml), leupeptin (50 μg/ml), pepstatin (1 μg/ml), and aprotinin (2 μg/ml)). The homogenates were centrifuged at 800 x g for 10 min at 4 ^{0}C, followed by centrifugation of the supernatants at 10,000 x g for 20 min at 4 ^{0}C to yield the crude cytosol and mitochondrial (pellet) fractions. Following centrifugation, the 10,000 x g crude mitochondrial pellet was resuspended in the homogenization buffer with proteinase inhibitors. Protein concentrations were determined by the BCA assay (Pierce, Lockford, IL). Aliquots of S10 or mitochondrial extracts, each containing 20 μg total protein, were loaded onto an 8-16% polyacrylamide SDS gel. The transfer membranes were probed with anti-human monoclonal cytochrome c, anti-human polyclonal AIF and β-actin antibodies at 1:1000 dilutions. Proteins were detected using horseradish peroxide-conjugated goat anti-mouse or anti-goat secondary antibodies (1:1000 dilutions) with the ECL-Plus reagent and Kodak X-OMAT film. The relative amounts of each protein were determined by counting the total number of pixels in each band (integrated density value, IDV) with a ChemiImager digital imaging system. The amount of a particular

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protein detected on the western blot was proportional to the total amount of protein loaded onto the electrophoresis gel. For the detection of soluble ICAD and cleaved ICAD, the S10 fractions were prepared as described above with the exception that the cells were lysed for 10 min on ice in buffer containing 10 mM HEPES-KOH, pH 8.0, 40 mM KCl, 3 mM $MgCl_2$, 10 % glycerol, 0.2 % Nonidet P40, 1mM dithiothreitol and protease inhibitor solution. Cytoplasmic extracts, containing either 20 μg or 40 μg of protein, were loaded on the electrophoresis gels and the blots were probed with anti-human ICAD antibody (1:500 dilution) or with GAPDH antibody (1:10,000 dilution). Proteins were detected with the ECL-Plus reagent and a Typhoon PhosphorImager (Amersham Biosciences Corp., Piscataway, NJ).

RESULTS

High Molecular Weight DNA Fragmentation Induced by Merbarone - Higher-order chromatin fragmentation (50-300 kb) induced by various apoptotic stimuli is thought to represent the excision of single DNA loops and rosettes of 2-6 loops from the nuclear matrix during the early stages of apoptosis (Alison and Sarraf, 1995; Pienta and Coffey, 1984; Lagarkova et al., 1995). We first examined the DNA fragmentation patterns induced by merbarone in HL-60 cells using a modified gel electrophoresis method (Barry and Eastman, 1993). This method involves in-gel digestion of cells with SDS and proteinase K, which avoids shearing and fragmentation of DNA during extraction. Incubation of the cells with 100 μM merbarone resulted in the generation of high molecular weight DNA in both a time- and dose- dependent manner (Figs. 1A and 1B). Apoptotic DNA ladders were detected after 6 h of merbarone treatment. The topoisomerase II cleavable complex forming drug, VM-26, also induced similar high molecular weight DNA fragmentation (Fig. 1A). Furthermore, the dose-dependent induction of DNA fragmentation by merbarone correlated with its inhibitory effects on the cell growth (Fig. 1B). The EC_{50} concentration for both inhibition of cell growth and formation of high molecular weight DNA fragments was about 70 μM.

Merbarone-Induced High Molecular Weight DNA Fragments Are Similar in Size to DNA Loops Released from the Nuclear Matrix - To determine whether the merbarone-induced high molecular weight DNA represent chromosomal DNA detached from the nuclear matrix, we used an *in vitro* assay

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for the isolation of DNA loops from the nuclear matrix (Solovyan et al., 2002). Agarose embedded cells were extracted with 1.5 M NaCl, washed, and then incubated in DNA cleavage buffer to form topoisomerase II cleavable complexes, followed by in-gel extraction with SDS to disrupt the topoisomerase II-DNA complexes and release the free DNA loops. Fig. 2A shows that incubation of the gel encapsulated nuclear matrix preparations with SDS resulted in the release of DNA loops that band at >50 kb, which is similar to the high molecular weight DNA fragments formed in merbarone-treated cells (Fig. 2B).

High Molecular Weight DNA Fragments Are Enriched In Nuclear Matrix Attachment Region Sequences - To provide further evidence that the merbarone-induced high molecular weight fragments are chromosomal DNA released from the nuclear matrix, we extracted prelabeled [^{14}C]DNA from the released high molecular weight DNA fragments and the bulk DNA that did not migrate into the separating gel. Equal amounts of [^{14}C]DNA were PCR amplified using primers specific for either a strong MAR in exon 3 of c-*myc*, or for a sequence in intron 1 of human β-globin that is transcriptionally inactive and does not contain either a MAR or a topoisomerase II consensus sequence (see Methods Section for a description of MARS and non-MARS sequences). The high molecular weight fragments from merbarone treated cells were enriched in the c-*myc* MARS sequence 321 ± 4 % (mean of 2 experiments ± range of extremes) (Fig. 3A, lane 3) compared to bulk DNA from untreated cells (Fig. 3A, lanes 1). In marked contrast, the high molecular weight DNA fragments from merbarone treated cells were depleted 87.1 ± 10 % in the non-MARS sequence in β-globin DNA (Fig. 3B, lane 3) relative to the bulk DNA from untreated cells (Fig.3B, lanes 1). Taken together, the results shown in Figures 2 and 3 indicate that the generation of high molecular weight DNA fragments induced by merbarone represents the release of chromosomal DNA from nuclear matrix. Our results are also consistent with published studies, which show that the higher-order chromatin fragmentation (50-300 kb) induced by other apoptotic agents represents the excision of single DNA loops and rosettes of 2-6 loops from the nuclear matrix (Pienta and Coffey, 1984;Alison and Sarraf, 1995; Lagarkova et al., 1995).

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Merbarone Inhibition of Topoisomerase II Does Not Directly Lead to Excision of High Molecular Weight DNA from the Nuclear Matrix - Studies were done to address the question whether inhibition of nuclear matrix topoisomerase II by merbarone directly releases the DNA loops from their anchorage sites on the nuclear matrix. Topoisomerase II-catalyzed cleavage of DNA loops was carried out in the absence of ATP using the gel encapsulated nuclear matrix preparations as described by Solovyan et al., (2002) and in Fig. 2. Fig. 4 indicates that when nuclear matrix preparations were incubated with either no drug (lanes 1-3) or with VM-26 (lane 5), in-gel digestion with SDS resulted in disruption of the topoisomerase II cleavable complexes and release of DNA loops. The DNA loops were released from the matrix preparations from control cells because the reactions lacked ATP, which prevented topoisomerase II from resealing the DNA breaks. However, merbarone blocked the release of the DNA loops greater than 96 ± 1.6 % S.E. (n = 4) compared to extracts not incubated with merbarone (lane 4 versus lane 1). These results are consistent with previous studies with cell free assays, which showed that merbarone inhibits topoisomerase II activity by blocking enzyme-mediated DNA cleavage and does not stabilize topoisomerase II cleavable complexes *in vitro* (Fortune and Osheroff, 1998). Thus, it appears that in HL-60 cells merbarone binding to topoisomerase II does not generate cleavable complexes that directly release high molecular weight DNA from the nuclear matrix.

Merbarone Induces a Rapid Loss of Mitochondrial Membrane Potential - We considered the possibility that inhibition of topoisomerase II catalytic activity by merbarone triggers apoptosis, and the excision of high molecular weight DNA from the nuclear matrix represents an initial DNA event in the apoptotic process. The dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$) is an early event in the mitochondrial pathway of apoptosis (Salvioli et al., 1997). To examine the temporal relationship between the onset of apoptosis and the formation of higher-order DNA fragments, HL-60 cells were treated with 100 μM merbarone and the $\Delta\Psi_m$ was monitored by flow cytometry over a 3 h period using the potential-sensitive cationic dye, JC-1. Data presented in Fig. 5 shows a rapid and extensive loss of $\Delta\Psi_m$ within 30 min after merbarone treatment. VM-26 (500 nM) also induced a nearly complete loss of

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mitochondrial membrane potential within 30 min (data not shown). Thus, the dissipation of $\Delta\Psi_m$ induced by merbarone preceded the formation of high molecular weight DNA fragments (Fig. 1). The rapid and nearly complete loss of mitochondrial membrane potential indicates that the induction of apoptosis by merbarone was not a cell cycle-specific effect.

Merbarone Induces Release of Mitochondrial Cytochrome C, but not AIF, into the Cytosol - Mitochondrial membrane permeabilization is thought to be the initial event that irreversibly commits the cell down the apoptotic pathway. Thus, we determined whether some of the known mitochondrial pro-apoptotic factors, in particular cytochrome c and AIF, are released into the cytosol after mitochondrial membrane depolarization. Fig. 6 shows the results of immunoblotting analysis of S10 cytoplasmic extracts and the mitochondrial fraction after merbarone treatment for 0 to 6 h. AIF remained in the mitochondrial fraction for up to 6 h after merbarone treatment, while release of cytochrome c into the cytosol was observed as early as 2 h after merbarone exposure. When the experiment was repeated similar results were obtained, i.e. cytochrome c but no detectable AIF was released into the cytosol of cells incubated with merbarone.

Merbarone-Induced Release of High Molecular Weight DNA Fragments Results from Activation of Caspase-Activated DNase (CAD) - Once released into the cytosol, cytochrome c can form complexes with Apaf-1, dATP or ATP, caspases-9 and caspase-3. This multimeric complex has been termed the apoptosome, which processes caspase-3 to its active form. Merbarone was previously shown to activate caspase-3 in CEM leukemia cells (Khélifa and Beck, 1999). Caspase-3 cleavage of ICAD releases active CAD, which then catalyzes the formation of oligonucleosome DNA ladders during the final stages of apoptosis. Recent evidence also suggests that CAD activity is responsible for the initial apoptotic cleavage of DNA into 50-300 kb fragments (Sakahira et al., 1999; Widlak, 2000). To investigate whether CAD is involved in merbarone-induced higher-order chromatin fragmentation, we preincubated cells with the pan-caspase inhibitor, z-VAD-fmk, prior to incubation with merbarone in order to prevent caspase activation. Preincubation of cells with z-VAD-fmk almost completely inhibited high molecular weight DNA fragment formation as well as the DNA laddering for as long as 6 h after merbarone treatment (Fig.

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7). Scanning of the gels from two separate experiments yielded the following degrees of inhibition induced by z-VAD-fmk compared to the corresponding untreated control samples: 4 h = 91 ± 0.07 % (range); 5 h = 95 ± 6.45 %; 6 h = 92 ± 14 %. We next examined the merbarone-induced activation of CAD by determining the cleavage of its inhibitor ICAD, which is required for CAD activation (Enari et al., 1998). Immunoblotting of the S10 cytoplasmic extracts (20 μg protein) from merbarone-treated cells revealed a time-dependent decrease in the amount of full-length ICAD (45 kDa) beginning at about 2 h, as well as a decrease in the amount of its smaller isoform (36 kDa) compared to extracts from 8 h control cells (Fig 8A). In addition, two cleavage products of ICAD were detected in merbarone-treated cells beginning at about 4 h when 40 μg of protein per lane was analyzed (Fig 8B). No ICAD cleavage was detected in control cells incubated with merbarone solvent for as long as 8 h. Similar results were obtained when the experiment was repeated. Thus, CAD activation coincided with the appearance of the high molecular weight DNA fragments (Fig. 1).

To further investigate the role of CAD in merbarone-induced DNA fragmentation, we used caspase-resistant Jurkat cells (ICAD/DM) that express a mutant form of ICAD (Sakahira et al., 1998; Sakahira et al., 1999). The ICAD mutant form carries a double mutation at the cleavage sites of caspase-3, thus rendering the ICAD/DM transformant resistant to caspase-3 and preventing subsequent release and activation of CAD. If CAD is important in merbarone-induced higher order DNA fragmentation, then the release of high molecular weight DNA fragments should be inhibited in the ICAD/DM transformant. A 5 h incubation of Jurkat cells expressing either vector only, pBabe, or ICAD/DM reduced subsequent cell growth to 50 and 40 % of control, respectively (data not shown). We then examined the DNA fragmentation pattern in these cells following treatment with 100 μM merbarone for 0 to 12 h. The results shown in Figs. 9A and 9B demonstrate that there was both a delay in the time of onset and a substantial reduction in the amount of merbarone-induced high molecular weight DNA fragmentation in the ICAD/DM transformants compared to the vector only pBabe cells. At 12 h after the start of merbarone treatment the amount of high molecular weight fragments was still reduced about 80% in the ICAD mutant. Oligonucleosomal DNA ladder formation was also prevented in the caspase-resistant

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ICAD/DM Jurkat cells (data not shown). These data together with the inhibition of high molecular weight DNA fragmentation by the caspase inhibitor, z-VAD-fmk (Fig.7), and the cleavage of ICAD (Fig. 8) indicate that merbarone-induced excision of high molecular weight DNA from the nuclear matrix requires CAD activity.

Decreased Formation of High Molecular Weight DNA Fragments in Topoisomerase II Deficient VM-1/C2 Cells Incubated with Merbarone- The initial apoptotic signal generated following inhibition of topoisomerase II by merbarone has not yet been identified. However, our data are consistent with the idea that merbarone-induced inhibition of topoisomerase II activity, as opposed to a direct effect of merbarone on mitochondria, is necessary to activate the apoptotic pathway. VM-1/C2 cells, a cloned subline of CEM/VM-1 cells that does not show impaired uptake of topoisomerase II-active agents (Danks et al., 1987), were 63-fold resistant to VM-26 (Lambert and Fernandes, 2000) and 17-fold resistant to merbarone (data not shown) based on the growth inhibition following a 48 h exposure to the drugs. The amount of nuclear matrix topoisomerase IIα in the resistant cells averaged 11 % of that detected in parental CEM cells (Lambert and Fernandes, 2000). As shown in Figure 10, CEM cells incubated with 100 μM and 200 μM merbarone for 24 h showed 8-fold and 20-fold greater formation of high molecular weight DNA fragments, respectively, compared to drug-resistant VM-1/C2 cells incubated with the same concentrations of merbarone. Similarly, the formation of high molecular weight DNA fragments was nearly completely inhibited in VM-1/C2 cells incubated for 24 h with 500 nM VM-26 (data not shown). Thus, these results suggest that inhibition of topoisomerase II activity by merbarone is necessary to generate the apoptotic signal that leads to the release of high molecular weight DNA from the nuclear matrix.

DISCUSSION

The cytotoxic effects of cleavable complex-forming topoisomerase II poisons, such as VM-26 and *m*-AMSA, are thought to result from the induction of DNA damage as opposed to simple inhibition of topoisomerase II enzymatic activity (Fernandes et al., 1988; Lambert and Fernandes, 2000). This has raised questions regarding the mechanisms by which catalytic inhibitors of topoisomerase II can kill

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tumor cells without inducing extensive DNA damage. In the studies reported herein, merbarone inhibited topoisomerase II catalyzed DNA cleavage in nuclear matrix preparations and blocked the release of DNA loops from the nuclear matrix. These results obtained with isolated nuclear matrix preparations are in agreement with previous studies with cell free assays, which showed that merbarone and other catalytic inhibitors of topoisomerase II block enzyme-mediated DNA cleavage (Fortune and Osheroff, 1998). However, the present study also indicates that in intact cells having a functional, mitochondrial apoptotic pathway, merbarone can promote extensive disorganization of DNA by initiating the apoptotic release of high molecular weight DNA fragments from the nuclear matrix. This type of DNA damage would not be obvious using conventional *in vitro* topoisomerase II-DNA cleavage assays or in alkaline elution assays of whole cells. Others have also reported that merbarone and other catalytic inhibitors of topoisomerase II (bisdioxopiperazines) induce various chromosomal aberrations (Stanulla et al., 1997; Wang and Eastmond, 2002).

Several lines of evidence from the present study and those from other laboratories are highly consistent with the concept that merbarone does not directly induce the release of DNA loops from the nuclear matrix by promoting topoisomerase II catalyzed DNA cleavage. Instead, merbarone can induce DNA fragmentation indirectly by triggering the mitochondrial apoptosis pathway, which leads to activation of the caspase-activated DNase, CAD. CAD activity appears responsible for the early apoptotic cleavage of DNA at the attachment sites of the DNA loops to the nuclear matrix. In support of this hypothesis, we have shown that the high molecular weight DNA fragments observed in merbarone-treated cells are enriched in aMAR sequences compared to bulk DNA. Furthermore, DNA fragmentation was preceded by cytochrome c release from the mitochondria, caspase activation of CAD, and was inhibited in caspase-3 resistant ICAD mutant cells. It was previously reported that incubation of isolated nuclei with activated CAD results in the excision of 50-300 kb DNA fragments prior to the appearance of oligonucleosome laddering (Widlak, 2000). Consistent with this, higher-order DNA fragmentation was blocked in caspase-resistant ICAD mutant cells (Sakahira et al., 1999).

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Critical insights into the possible mechanisms by which catalytic inhibitors of topoisomerase II induce higher-order chromatin fragmentation are provided by the studies of Durrieu *et al.* (2000) and Widlack *et al.* (2000). These investigators showed that in cells treated with catalytic inhibitors of topoisomerase II, the DNA cleavage activity of CAD is enhanced following its binding to topoisomerase II. The DNA cleavage was accompanied by apoptotic chromatin condensation. Since topoisomerase IIα is enriched at the bases of the matrix-bound DNA loops compared to nonmatrix DNA (Danks et al., 1994), this protein could function to recruit CAD to the DNA loop attachment sites on the matrix and stimulate CAD endonuclease activity. Beere *et al.* (1995) suggested that the proteolytic degradation of nuclear matrix topoisomerase II observed following VM-26 induced apoptosis of leukemic cells may be responsible for the higher-order DNA fragmentation by exposing the DNA-matrix attachment regions to other nucleases. Our data and those of others do not support this mechanism, since DNA fragmentation was greatly reduced in ICAD mutants. Huang *et al.* (1997) reported that various cysteine protease inhibitors including Z-VAD blocked the drug-induced generation of 50-300 kb DNA fragments in whole cells. However, these protease inhibitors did not decrease the formation of 50-300 kb fragments in isolated nuclei incubated with extracts from cells undergoing drug-induced apoptosis. These results indicate that cysteine proteases are not directly involved in higher-order chromatin fragmentation but act upstream (e.g. caspase activation of CAD) from the endonucleolytic cleavage of DNA loops from the nuclear matrix.

Our results may also provide an explanation for the observation that merbarone is a potent inducer of cleavage in the breakpoint cluster region of the *MLL* gene on chromosome 11q23 (Stanulla et al., 1997). Secondary leukemias that develop following cancer chemotherapy, especially those induced with topoisomerase II-active drugs, are frequently associated with site-specific breakage within the *MLL* bcr. It has been proposed that drug-induced cleavage within the MLL bcr is mediated by an apoptotic nuclease (Sim and Liu, 2001). Since this bcr contains topoisomerase II consensus sequences and a MAR (Stanulla et al., 1997), this region is likely a sensitive target for the merbarone-induced cleavage of matrix-attached DNA during the initial stages of apoptosis. These observations suggest that although

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merbarone does not form cleavable complexes with topoisomerase II, it still has the potential to induce secondary leukemias.

The results reported herein suggest that inhibition of topoisomerase II activity by merbarone activates a mitochondrial pathway of apoptosis, and may not result from a direct effect on mitochondria. Topoisomerase II-deficient VM-1/C2 cells incubated with merbarone showed much less formation of high molecular weight DNA fragments than parental CEM cells incubated with the same concentrations of merbarone. It has been proposed that the mitochondrial permeability transition pore complex (PTPC) is a sensor of intracellular damage (Yang and Cortopassi, 1998). Thus, inhibition of topoisomerase II activity by merbarone or other agents could lead to alterations in the cellular ATP/ADP ratio, redox potential, mitochondrial membrane potential etc, which could activate the PTCP and release cytochrome c from the mitochondria. Additionally, it has been reported that merbarone rapidly induces JNK signaling (Khélifa and Beck, 1999), and this event may be responsible for activation of the mitochondrial apoptotic pathway.

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FIGURE LEGENDS

Fig. 1. Merbarone-induced high molecular weight DNA fragmentation. ***A***, Time-course of drug-induced DNA fragmentation. HL-60 cells were incubated with either 100 μM merbarone or 500 nM VM26 for the time periods listed at the top of each gel lane. DNA fragmentation was analyzed by a modified gel electrophoresis procedure, which included in-gel digestion of the cells with SDS and proteinase K. m, molecular weight marker. C, vehicle controls. Arrows indicate 50-300 kb fragments. ***B***, Effects of merbarone on growth inhibition and higher-order DNA fragment formation as a function of merbarone concentration. Cells were incubated with 0-100 μM merbarone and DNA fragment formation was measured 5 h later and cell numbers were counted 48 h after the addition of merbarone. Symbols: □, dosage effect of merbarone on cell growth; ●, dosage effect of merbarone on DNA fragmentation. Values are the means of three determinations ± standard error.

Fig. 2. Merbarone-induced high molecular weight DNA fragments are similar in size to DNA loops extracted from nuclear matrix. ***A***, HL-60 cells were incubated for 5 h with either 0 or 100 μM merbarone. The cells were encapsulated in 1% agarose and then extracted with 1.5 M NaCl. Topoisomerase II-linked DNA loops were subsequently released from the nuclear matrix by incubating the high salt-extracted cells in DNA cleavage buffer followed by in-gel digestion with SDS. m, molecular weight marker. ***B,*** 50-300 kb fragments from merbarone pretreated cells. The arrows indicate the released DNA loops (A) or 50-300 kb fragments induced by merbarone (B). Fig. 2 is an example from two separate experiments, each done in triplicate.

Fig. 3. MARS sequences are enriched in the merbarone-induced high molecular weight DNA fragments. HL-60 cells were incubated for 5 h with either 0 or 100 μM merbarone. ***A***, MARS sequences in either bulk DNA (lanes 1 and 2) or high molecular weight DNA fragments (lane 3) were amplified by PCR. The bulk DNA (lanes 1 and 2) and DNA fragments (lane 3) were analyzed by PCR using primers specific for a MAR in c-*myc* (A) or a region in β-globin DNA lacking a MAR sequence. ***B***, Equal

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amounts of prelabeled [^{14}C]DNA were amplified and analyzed on a 2 % agarose gel. Similar results were obtained when the experiment was repeated.

Fig. 4. Effects of topoisomerase II-active drugs on the release of DNA loops from the nuclear matrix. High-salt extracted nuclei from HL-60 cells were incubated with either DNA cleavage buffer alone (lane 1), the appropriate solvents for topoisomerase II drugs (lane 2 and 3), with 100 μM merbarone (lane 4) or with 20 μM VM-26 (lane 5) for 30 min at 37 ^{o}C. Released-DNA loops were analyzed by agarose gel electrophoresis, which included in-gel digestion with SDS. m, molecular weight marker.

Fig. 5. Induction of apoptosis in HL-60 cells by merbarone. The time course of induction of apoptosis by merbarone (100 μM) was followed by flow cytometry using the potential-sensitive dye, JC-1, to monitor changes in mitochondrial transmembrane potential. Values are the means of two experiments ± range of the mean of each experiment.

Fig. 6. Release of mitochondrial cytochrome c, but not AIF, into the cytosol in merbarone-treated HL-60 cells. After treatment of HL-60 cells with 100 μM merbarone for 0 to 6 h, the crude mitochondrial and S10 cytoplasmic fractions were prepared and aliquots of each fraction containing 20 μg of protein were loaded onto an 8 to 16% gradient polyacrylamide SDS gel. Immunoblotting was carried out using anti-human AIF, cytochrome c and actin antibodies. Similar results were obtained when the experiment was repeated.

Fig. 7. Inhibition of high molecular weight DNA fragmentation by z-VAD-fmk. HL-60 cells were preincubated with 20 μM z-VAD-fmk for 30 min at 37 ^{o}C prior to incubation with 100 μM merbarone for 4-8 h. DNA fragmentation was analyzed on a 1.5% agarose gel as before. C, vehicle control. The arrow indicates released high molecular weight DNA fragments.

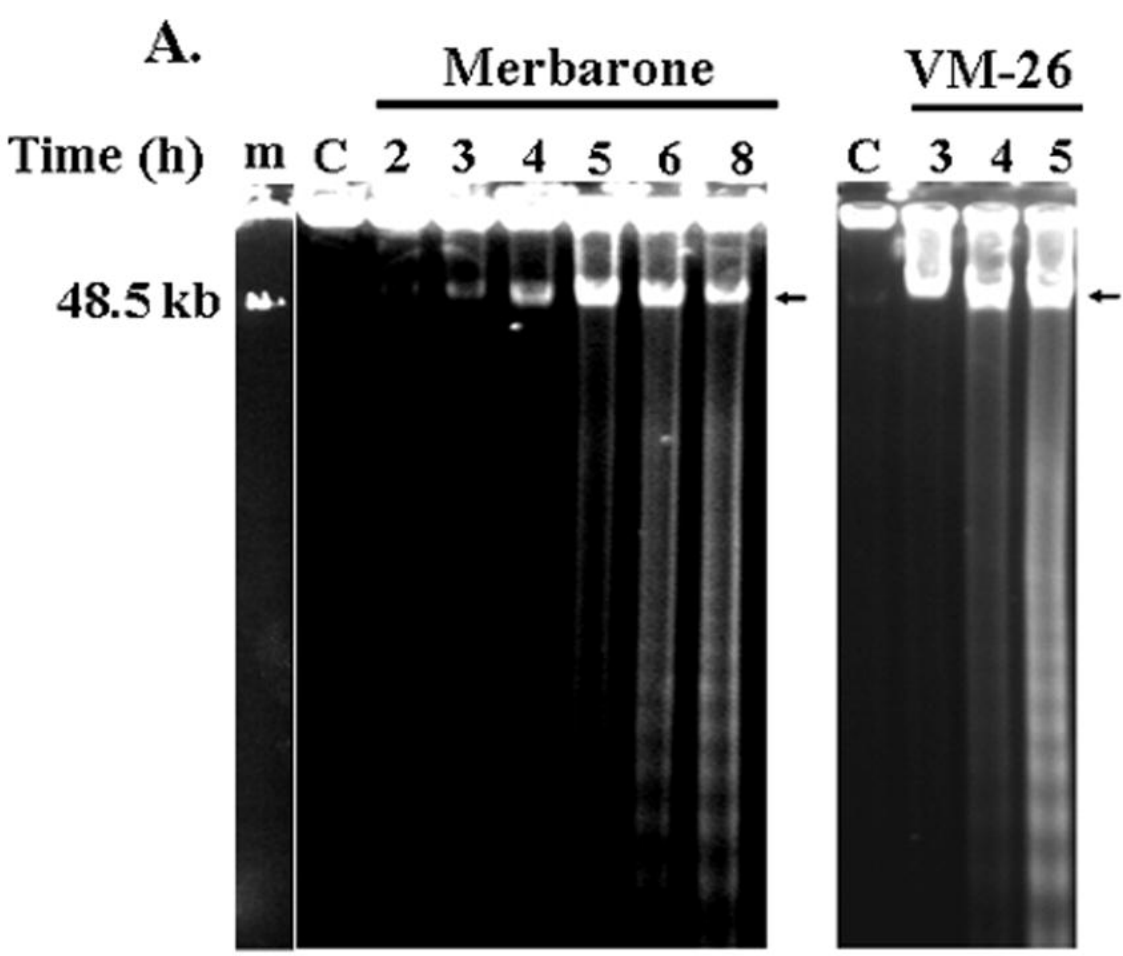
Fig. 8. Merbarone-induced activation of CAD. ***A***, HL-60 cells were incubated with 100 μM merbarone for 2-8 h and aliquots of the S10 fractions containing 20 μg of protein were loaded onto an 8 to 16% gradient polyacrylamide SDS gel. Immunoblotting was carried out using anti-human ICAD antibodies. ***B***, Similar to panel A with the exception that 40 μg aliquots were loaded onto the gel to

reveal the ICAD cleavage products. Lane C in panels A and B refers to cells incubated with the merbarone solvent for 8 h.

Fig. 9. Merbarone-induced higher molecular weight DNA fragmentation is inhibited in caspase resistant ICAD Jurkat cells - ***A***, Jurkat cells expressing a retroviral vector pBabe or caspase resistant ICAD double mutant (ICAD/DM) were incubated with either vehicle control (lanes 2 and 3 pBabe; lanes 10 and 11 ICAD/DM) or 100 μM merbarone (lanes 4-8 pBabe; lanes 12-16 ICAD/DM) for 0-12 h. Aliquots of 5.0 x 10^5 cells were digested in the agarose gel during electrophoresis and following electrophoresis the gel was stained with ethidium bromide. The high molecular weight DNA fragments were analyzed with a ChemiImager digital imaging system. ***B***, Quantitative representation of the amounts of high molecular weight DNA fragments. Filled and unfilled bars represent DNA fragmentation in pBabe or ICAD/DM Jurkat cells, respectively. Values are the average of 2 experiments ± range of the means of each experiment.

Fig. 10. High molecular weight DNA fragmentation in CEM and VM-1/C2 cells incubated with merbarone. ***A,*** CEM and VM-1/C2 cells were incubated for 24 h with various concentrations of merbarone from 0-200 μM. DNA fragmentation was analyzed as described in Figure 1A. Arrow indicates released DNA fragments. ***B,*** Quantitative representation of the amounts of high molecular weight DNA fragments. Filled and unfilled bars represent DNA fragmentation in CEM or VM-1/C2 cells, respectively. The results are the means of duplicate experiments and are expressed as the mean fold increase ± range in the amounts of high molecular weight DNA fragments in merbarone-treated CEM cells and VM-1/C2 cells compared to the untreated CEM and VM-1/C2 cells.

Figure 1


A.
Merbarone
VM-26
Time (h)
m
C
2
3
4
5
6
8
C
3
4
5
48.5 kb

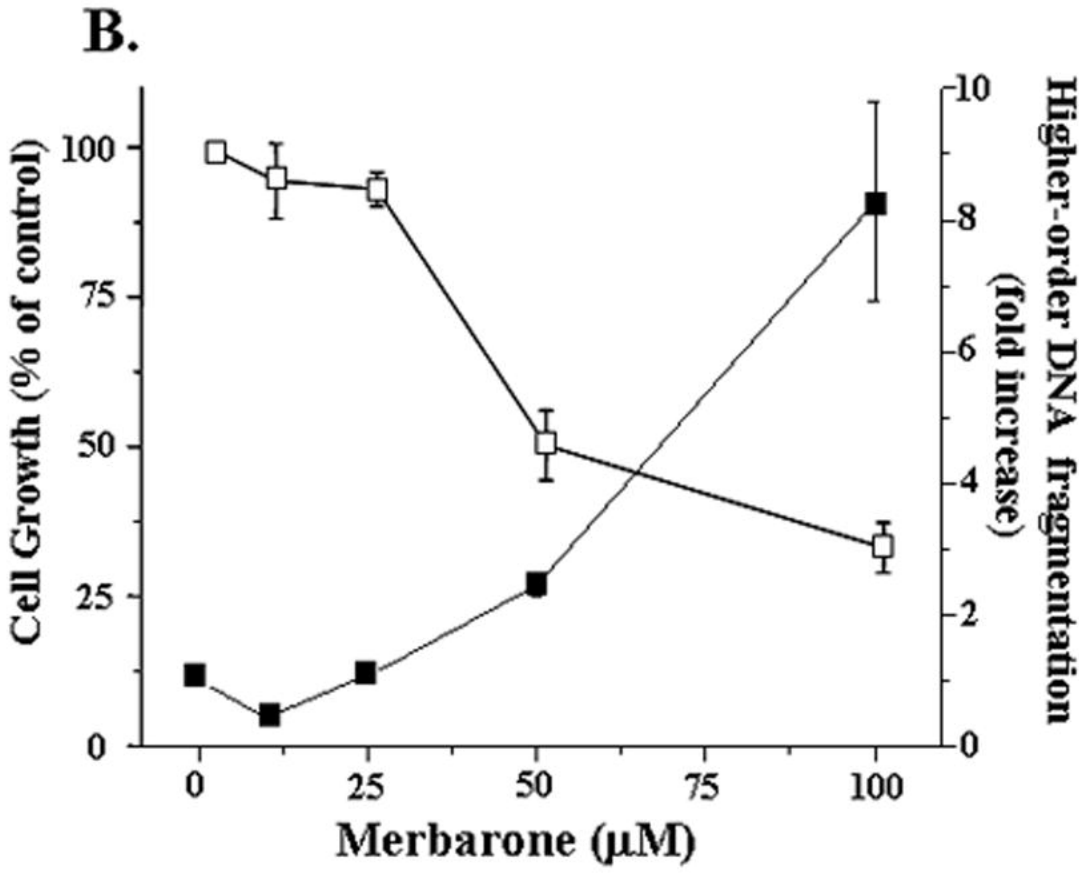

B.
Cell Growth (% of control)
Higher-order DNA fragmentation (fold increase)
Merbarone (μM)
0
25
50
75
100
0
2
4
6
8
10

Figure 2

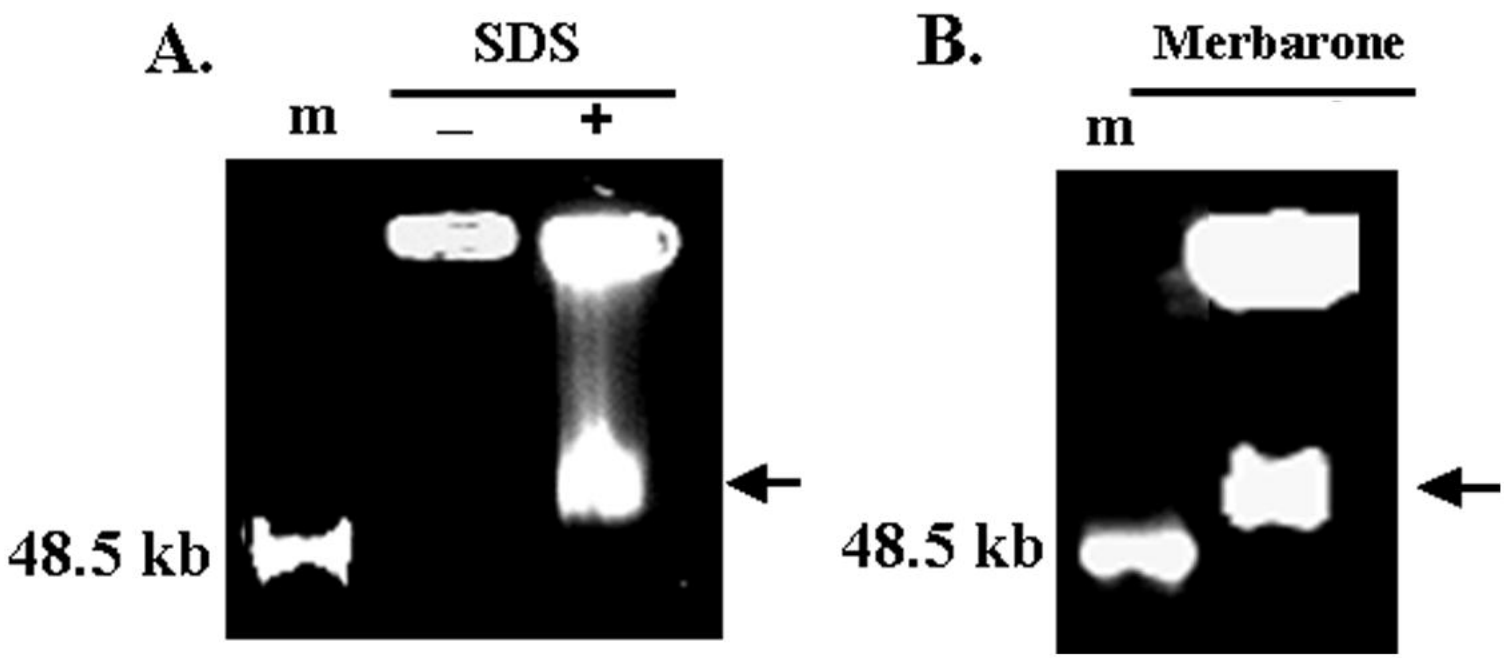


Figure 3

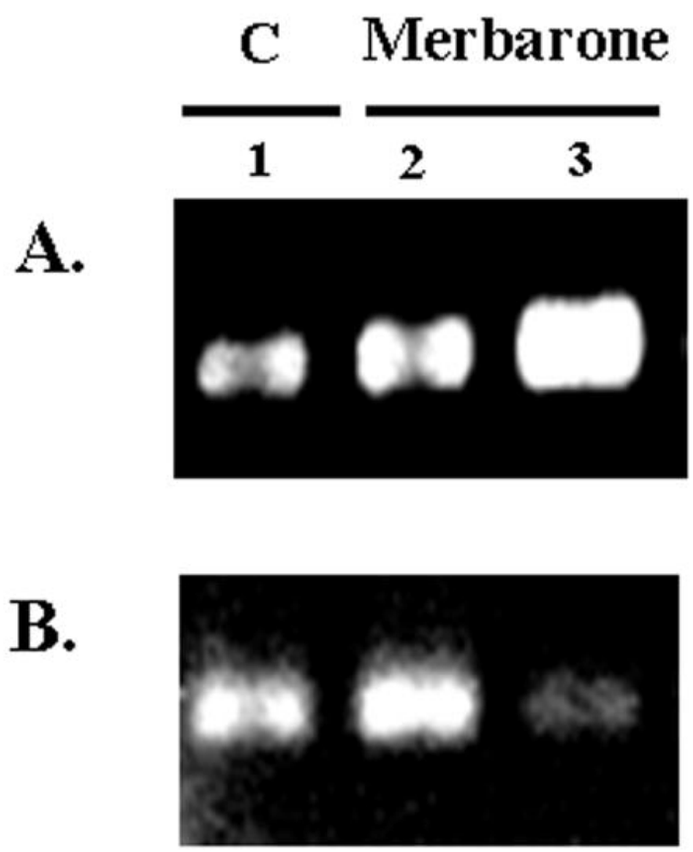


Figure 4

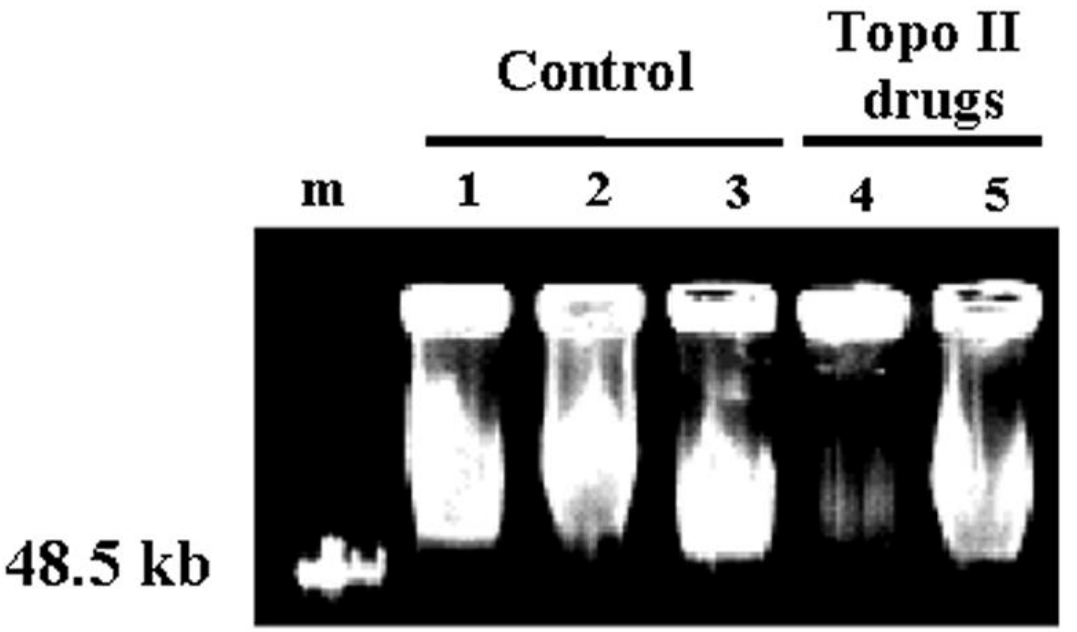


Figure 5

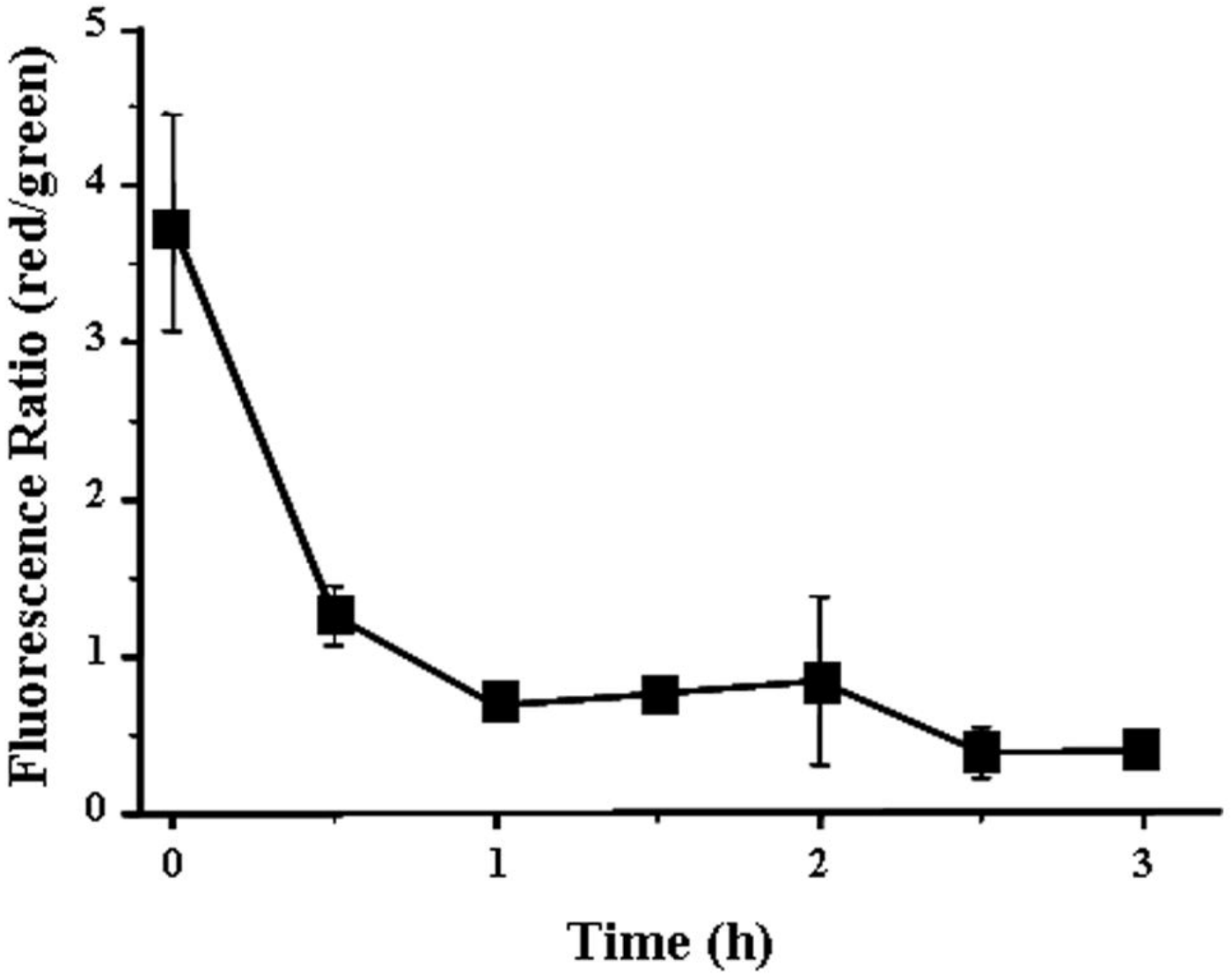


Figure 6

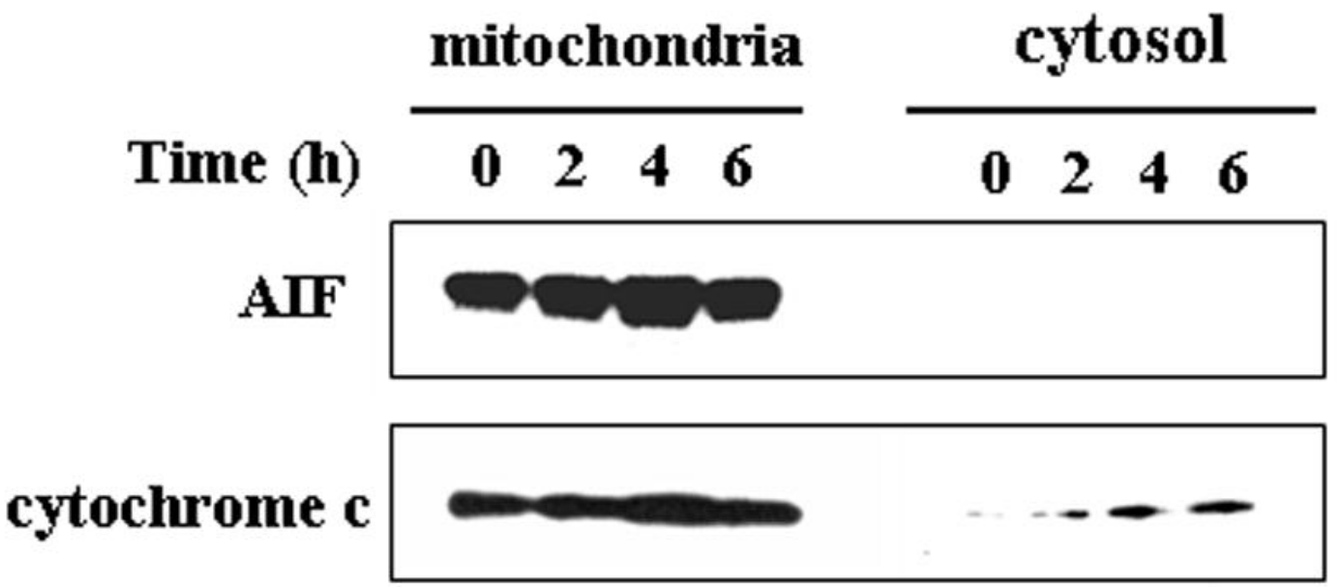


Figure 7

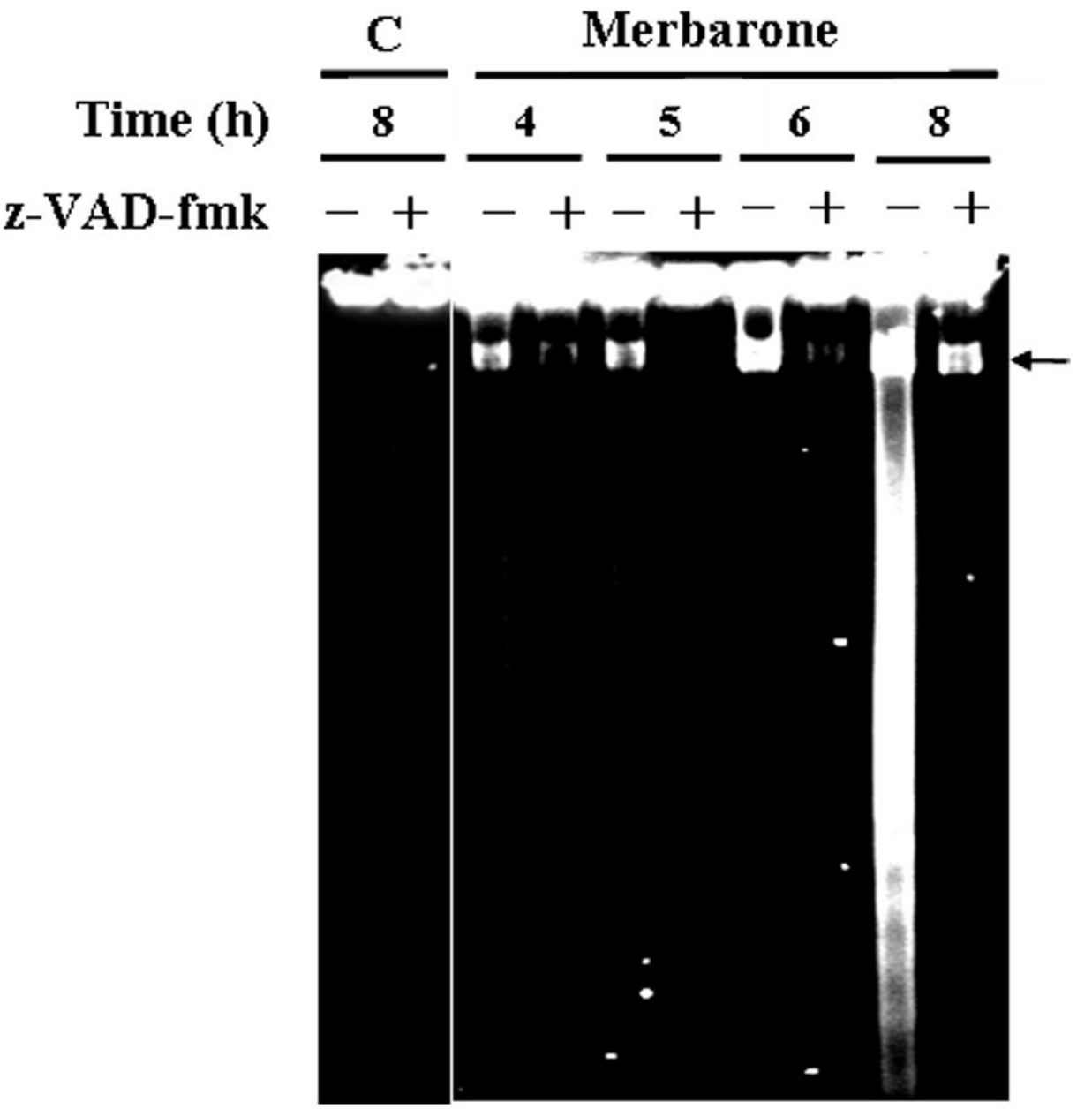


Figure 8

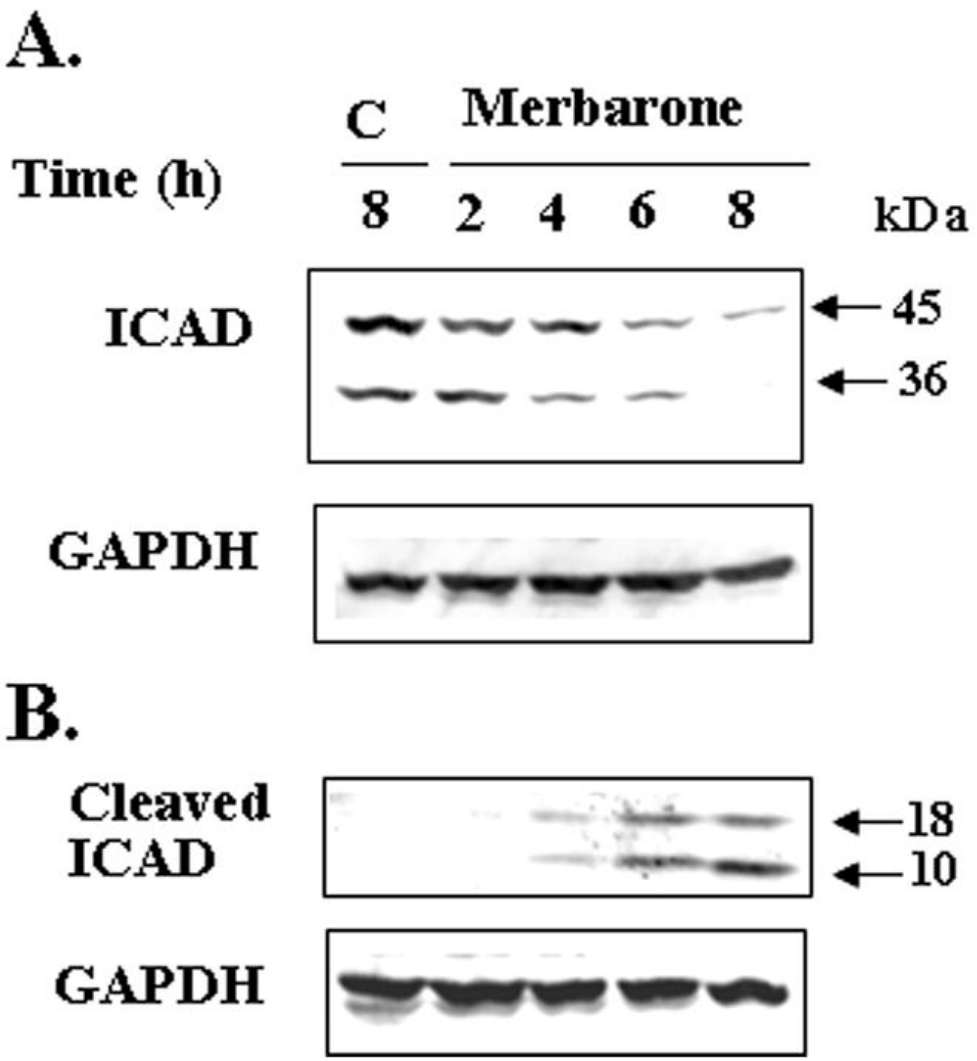

A.
C
Merbarone
Time (h)
8
2
4
6
8
kDa
ICAD
45
36
GAPDH
B.
Cleaved
ICAD
18
10
GAPDH

Figure 9

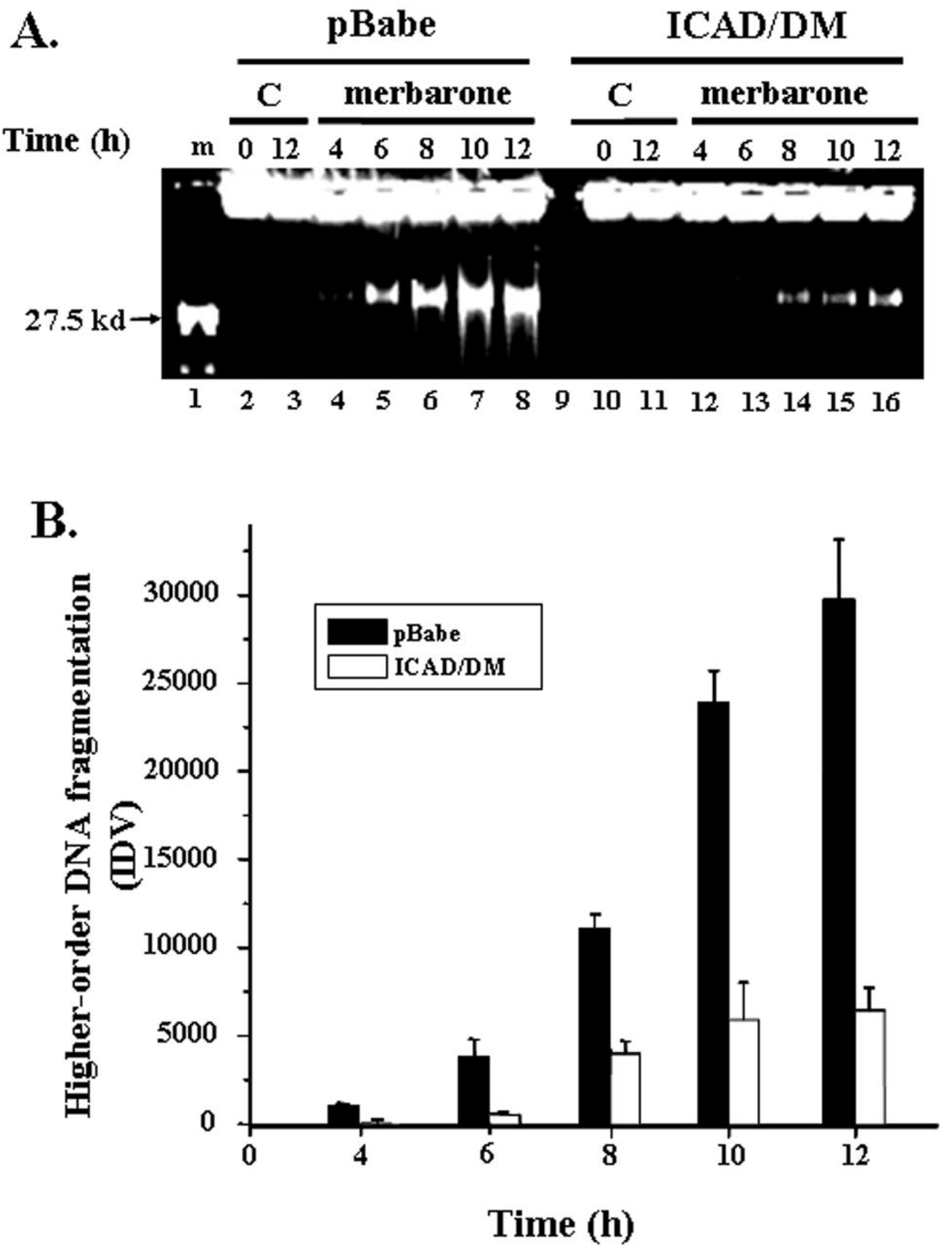
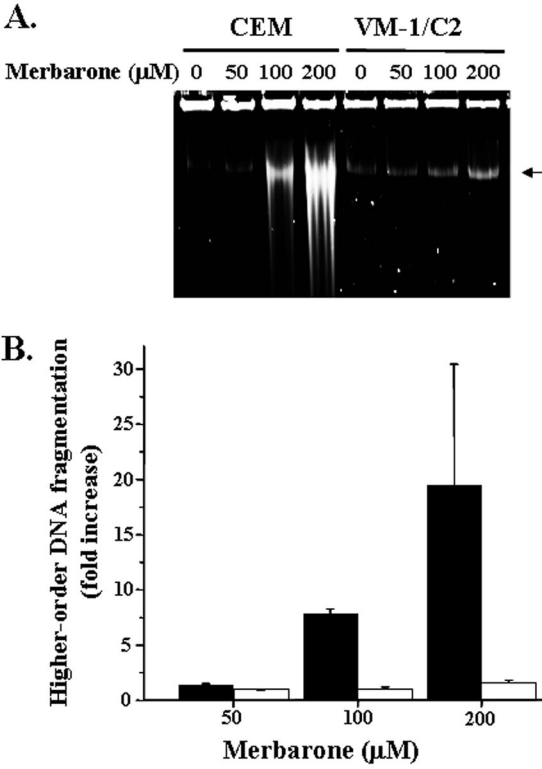

A.
pBabe
ICAD/DM
C
merbarone
C
merbarone
Time (h)
m 0 12 4 6 8 10 12
0 12 4 6 8 10 12
27.5 kd
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B.
Higher-order DNA fragmentation (IDV)
30000
25000
20000
15000
10000
5000
0
pBabe
ICAD/DM
0 4 6 8 10 12
Time (h)

Figure 10


A.
CEM
VM-1/C2
Merbarone (μM) 0 50 100 200 0 50 100 200
B.
Higher-order DNA fragmentation
(fold increase)
0
5
10
15
20
25
30
50
100
200
Merbarone (μM)