

PARP-inhibition potentiates apoptosis in p53 deficient cells

PARP INHIBITION SENSITIZES P53-DEFICIENT BREAST CANCER CELLS TO DOXORUBICIN-INDUCED APOPTOSIS.

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Abbreviations: ANI, 4-amino-1,8-naphthalimide; PARP, poly (ADP-ribose) polymerase ; PAR, poly (ADP-ribose) ; doxo, doxorubicin ; LD₅₀, Lethal dose 50 % ; CFA, Colony forming assay

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Abstract

P53 deficiency confers resistance to doxorubicin (doxo), a clinically active and widely used antitumour anthracycline antibiotic. The purpose of the current study was to investigate the reversal mechanism of doxorubicin resistance by the potent poly(ADP-ribose) polymerase (PARP) inhibitor 4-amino-1,8-naphthalimide (ANI), in the p53 deficient breast cancer cell lines EVSA-T and MDA-MB-231. The effects of ANI, in comparison with doxo alone, on doxo-induced apoptosis, were investigated in matched pairs of EVSA-T or MDA-MB-231 with or without ANI co-treatment. Doxo elicited PARP activation as determined by western blot and immunofluorescence of poly (ADP-ribose) and ANI enhanced doxo cytotoxic activity 2.3 times and in a caspase-dependent manner. The long-term cytotoxic effect was studied by colony forming assay. Using this assay ANI also significantly potentiates the long term cytotoxic effect respect to treatment with doxo alone. Drop in mitochondrial potential together with an increase in cytochrome c release, association of Bax to the mitochondria and caspase-3 activation were also observed in the presence of ANI. Therefore, PARP inhibition may represent a novel way of selectively targeting p53 deficient breast cancer cells. The underlying mechanism is probably a potentiation of unrepaired DNA damage, shifting from DNA repair to apoptosis due to the effective inhibition of PARP activity.

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Introduction

Dysregulation of normal apoptotic mechanisms provides a growth advantage to cancer cells [1]. In breast cancer, dysregulated apoptotic pathways include down-regulated death receptor pathway function, p53 mutations, and abnormal bcl-2 pathway function [2-4]. Furthermore, breast cancer treatments including chemotherapy, radiation therapy and hormone therapy induce apoptotic mechanisms to cause cancer cell death [5]. Therefore, activation of specific apoptotic mechanisms in breast cancer cells could be an effective means to treat breast cancer.

Poly(ADP-ribose) polymerase-1 (PARP-1) is the principal member of a family of enzymes possessing poly(ADP-ribosylation) catalytic capacity. Is a conserved nuclear protein that binds rapidly and directly to both single and double-strand breaks. Both processes activate the catalytic capacity of the enzyme which in turn modulates the activity of a wide range of nuclear proteins by covalent attachment of branching chains of ADP-ribose moieties. Organisms and cellular systems deficient in functional PARP-1 display severely impaired base excision repair and genomic instability, suggesting that the enzyme may play a primary role in the cellular response to DNA damage [6].

Increasing interest in potential clinical applications of PARP inhibition has led to the development of a wide range of new compounds, the more recently developed of which display greatly increased potency and specificity compared with the prototype PARP inhibitor, 3-aminobenzamide (3-AB) [7]. In particular, higher potency PARP inhibitors have a greatly reduced effect on mono(ADP-ribosyl) transferase enzymes. 4-amino-1,8-naphthalimide (ANI) is a potent PARP inhibitor ($IC_{50} = 180$ nM) and has been reported to increase the sensitivity to radiation in a number of human tumour cell lines, both in vitro and when grown as xenografts in mice [8]. Apoptosis is one of the most important pathways through which chemotherapeutic agents inhibit the growth of cancer cells. Thus, it is crucial to investigate whether the induction of apoptosis is associated with the molecular mechanism by which inhibition of PARP may exert its biological effects on breast cancer cells.

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The objectives of the present study were to investigate whether ANI could potentiate the cytotoxic effect of doxorubicin in p53-deficient human breast cancer cell lines, EVSA-T and MDA-MB-321, and elucidate the molecular mechanism by which ANI and doxo may induce apoptotic cell death in this cell lines. Our data show that doxo induce a rapid PARP activation and moderate cell killing which is markedly potentiated by co-treatment with the PARP inhibitor ANI by accelerating the mitochondrial steps of apoptosis. In summary, our data suggest that PARP inhibition may represents a novel way of selectively targeting p53 deficient breast cancer cells.

Experimental

Cell culture. EVSA-T and MDA-MB-231 cells (breast cancer cell lines with p53 mutated [9, 10]) were maintained in DMEM supplemented with 10 % FBS at 37 °C in a humidified 5% CO₂ atmosphere. Cells were plated 24 h prior to doxo treatment.

Drugs. Cells were treated with doxo for 1 hour in DMEM supplemented with 10 % FBS at 37 °C in a humidified 5% CO₂ atmosphere. The PARP inhibitor 4-amino-1,8-naphthalimide (ANI 10 µM) was dissolved in culture medium immediately before use. ANI solutions (10 µM), contained < 2% DMSO in addition to improve solubility. ANI is sparingly soluble in water without adding DMSO. ANI was added 1 hour prior to doxo treatment and thereafter present in the culture throughout the experiment. The pan-caspase inhibitor z-VAD-FMK (z-Val-Ala-Asp-(oMe)-FMK) (50 µM) was added 2 hours prior to doxo treatment and was thereafter present in the culture throughout the experiment.

Analysis of cell death. Cell viability was evaluated as previously described by the sulphorhodamine B method [11]. Measurement of apoptosis was determined by Annexin V staining. Following drugs treatments, cells were harvested using trypsin-EDTA, washed once in ice cold PBS and resuspended in 1 ml Annexin V binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM

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CaCl₂). 75.000 cells were stained with 5 µl Annexin V FLUOS (Roche Molecular Biochemicals) in 100 µl Annexin V buffer at 4 °C. After 30 min, 100 µl binding buffer was added to each tube and samples were analyzed using a tri-laser FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

Sub-G₁ analysis was examined by flow cytometry using the propidium iodide (PI) DNA staining method. Cells were harvested with trypsin-EDTA, washed once in ice cold PBS and re-suspended in 100 µl of PBS. 900 µl of ice cold ethanol 70% was added to the cells during 5 min, washed with 2 ml of PBS and the cells were re-suspended in 250 µl of PI/RNase solution (PBS, 100 µg/ml RNase, 40 µg/ml PI). After 30 min, samples were analyzed using a tri-laser FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

Colony forming assay (CFA). Semi-confluent culture flasks were trypsinized, and the adequate cell number were seed in 25 cm² tissue culture flasks. One day later, cells were exposed to doxo at the indicated concentrations for 1 hour. Cells were treated with the PARP inhibitor ANI (10 µM) for 24 hours. Fifteen or twenty days later, cells were stained with crystal violet and colonies of 50 or more cells were scored. Surviving fractions were determined from colony counts and were corrected for the plating efficiency of the non-treated controls.

Immunofluorescence. Immunostaining for poly(ADP-ribose) (PAR) was performed on cells grown on glass cover-slips and fixed in ice-cold methanol-acetone (1:1) for 10 minutes. Poly(ADP-ribose) was detected by immunofluorescence, using monoclonal antibody 10H (Alexis, Germany) and FITC-conjugated goat anti-mouse immunoglobulins (Sigma, St Louis Mo). Nuclear counterstaining with propidium iodide was performed after removal of excess secondary antibody. Immunostaining was visualized with a Leica Spectral confocal laser microscope.

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Western blot analysis. Cells were detached from the culture flask, washed with PBS and resuspended in 100 μ l lysis buffer (50 mM Tris-HCl pH 8, 0.1 mM EDTA, 0.5% Triton X-100, 12.5 mM β -mercaptoethanol) for 30 minutes on ice. Pellet was eliminated and sample buffer (50 mM Tris-HCl pH 6.8, 6 M urea, 6% β -mercaptoethanol, 3% SDS, 0.003% bromophenol blue) was added to the supernatant. Proteins were resolved on SDS-12% polyacrylamide gels and transferred onto Immun-Blot PVDF Membrane (Biorad). The blot was blocked with 5% milk powder in PBS with 0.1% Tween-20 for 30 minutes, washed with PBS/Tween, and incubated overnight with antibodies anti-poly(ADP-ribose) (PAR) (BIOMOL), anti-PARP-1 (EGF-69), anti-Bax and anti-cytochrome c (Pharmingen, San Diego, CA, U.S.A.), anti- α -tubulin (Sigma, St Louis Mo), and 2 hours with appropriate secondary antibodies. Bands were visualized by ECL-PLUS (Amersham Biosciences) and the pictures were taken with the imaging system ChemiDoc XRS System (BIO-RAD).

For measurements of cytochrome c and Bax, cells were washed with PBS and lysed for either 5 min in 30 μ l of ice cold lysis buffer (80 mM KCl, 250 mM sucrose, 500 μ g/ml digitonin, 1 μ g/ml of each of the protease inhibitors: leupeptin, aprotinin, pepstatin, and 0.1 mM PMSF in PBS). Then, cell lysates were centrifuged for 5 min at 10,000 xg . Proteins from the supernatant (cytosolic fraction) and pellet (membrane fraction) were mixed with sample buffer and resolved on SDS/polyacrylamide (12%) gels. Cytochrome c and Bax were determined by Western-blot analysis as described above.

Mitochondrial membrane potential detection. Cells were plated in 6-well plates (2×10^5 cells) and grown for 24 hours. After this period, the cells were treated with doxo (1 μ g/ml) and doxo (1 μ g/ml) plus ANI (10 μ M). Cells were harvested using trypsin-EDTA, washed once in ice cold PBS and re-suspended in collected in PBS with DIOC₆ (40 nM) for 30 min at 37 °C. Samples were then analysed with FACScan cytometer (Becton-Dickinson) and the fluorescence was detected in FL1. During analysis of the flow cytometric data, a marker indicating a cell population with lower $\Delta\psi_m$ was applied to histograms, and the percentage of the cells in the region was determined. The numbers of cells with

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lower membrane potential (cells treated with doxo, with or without ANI) were represented as a percentage of cells without treatment (control cells).

Electrophoretic mobility shift assay (EMSA). The double-stranded oligonucleotides 5'- TGCTAGGGGGATTTTCCCTCTTCTTGT-3' [12] with the sequences of the binding sites for NF- κ B of iNOs promoter were purchased. These oligonucleotides were end-labelled with [γ - 32 P]ATP and T4 DNA polynucleotide kinase and used as probe. Nuclear extracts were obtained according to a previous report [13]. Nuclear extracts (3 μ g protein) were incubated with 2 μ l of 32 P-labelled probe (6 X 10⁴ d.p.m) in a final volume of 20 μ l of reaction mixture for 15 min at 4 °C as described previously [14]. The DNA-protein complexes were separated on native 5% polyacrylamide gels in 0.5 Tris-borate EDTA buffer.

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Results

Doxo treatment induces PARP activation. Many different cell insults infringing DNA damage, have been shown to be able to activate PARP. Doxo is a powerful DNA damaging agent but there are no evidences in the literature of a direct effect on PARP activation in tumour cells. We hypothesized that the activation of PARP may counteract the doxo-induced cytotoxicity by promoting DNA repair. First we aim to demonstrate that doxo-induced PARP activation. In figure 1 we show that doxo (1 μ g/ml) is able to induce a rapid PARP activation (20 minutes after treatment) measured both by western blot (figure 1A (i) (EVSA-T) and (ii) (MDA-MB-231)) or immunofluorescence (figure 1B, EVSA-T cells). Pre-treatment with 10 μ M ANI completely prevented doxo-induced PARP activation (figure 1A (ii) and 1B (iv)). The ability of ANI as a PARP inhibitor has been extensively described elsewhere [15].

Co-treatment of p53-deficient breast cancer cells with doxo + ANI potentiates apoptotic cell death. EVSA-T breast cancer cells are resistant to treatment with a number of chemotherapeutic agents, including doxo [9]. In a preliminary assay we did a dose response of doxo-induced cell death to choose at which dose these cells start to be sensitive to the drug. In short-term experiments of cell viability (until 72 hours) they were completely resistant to doses below 1 μ g/ml and partially sensitive to 1 μ g/ml, reaching a 50% of cell death after doxo treatment (figure 2A). These results show that EVSA-T cells were very poorly sensitive to doxo-induced cytotoxic effect, as has been previously shown [11]. Then we used annexin V and sub G1 to evaluate cell death as a measure of short-term, and colony forming assay (CFA) as a measure of long-term cytotoxic effect and we also studied the effect of ANI co-treatment in a second p53-deficient breast cancer cell line ,MDA-MD-231, to further substantiate our observation. Pre-treatment with 10 μ M ANI resulted in a potentiation of doxo-induced cell death measured by all three criteria (Figure 2B (annexin V in EVSA-T), 2C (sub-G₁ in EVSA-T), 2D (sub-G₁ in MDA-MB-231) and 2E (CFA in EVSA-T)). The extent of potentiation of cell death was 2.3 fold with annexin V (figure 2B) and 1,7 fold and 2 fold respectively with sub G₁ (figure 2C and D) . Long-term cytotoxicity using ANI and doxo was also

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potentiated respect to treatment with doxo alone according to the CFA (figure 2E). The effect of ANI was completely abolished with the pan-caspase inhibitor Z-VAD-fmk (figure 2B), suggesting that ANI was activating the apoptotic pathway at some point.

In most cases, p53-induced apoptosis proceeds through translocation of the cytoplasmic protein Bax to the mitochondria where it co-operates with truncated Bid in the release of cytochrome *c*, leading to caspase activation. This pathway is impaired in p53 mutant cells like EVSA-T. To more precisely analyze which steps of apoptosis were altered by ANI, we studied depolarization of mitochondrial membrane potential, mitochondrial bax translocation, cytochrome *c* release and activation of caspase-3 by PARP-1 cleavage (which have been described as hallmark of doxo-induced apoptosis) in EVSA-T cells. In figure 3A, B and C the change in mitochondrial permeability was poorly shifted by doxo alone, and the pre-treatment with ANI further increased the drop in permeability. This change was also accompanied by an increase in bax migration, cytochrome *c* release (figure 3D) and caspase-3 activation, as measured by PARP-1 cleavage (figure 3E), suggesting that PARP inhibition is able to restore a p53-like response in these cells.

Another mechanism by which tumour cells may be resistant to chemotherapy is the activation of the transcription factor NF- κ B, which is responsible for the activation of anti-apoptotic genes [16] and has been involved in the progression to hormone independence in breast cancer [17]. Several laboratories, including ours, have shown that elimination of PARP-1 impairs NF- κ B response [13],[14]. We have evaluated the impact of ANI on doxo-induced NF- κ B activation by EMSA and we found that ANI do not affect the ability of doxo to induce NF- κ B activation, suggesting that ANI-induced potentiation of cell death is independent of NF- κ B (figure 4).

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Discussion

A panel of biological markers including regulators such as p53, Bcl-2 family proteins, caspases and DNA fragmentation factor have been described as having a role in apoptosis. The assessment of these in cell lines and in clinical samples, particularly in the neo-adjuvant setting, help to build a picture of their contribution to the biology of chemo-resistance.

EVSA-T and MDA-MB-231, breast cancer-derived cell lines, are deficient in p53 and relatively insensitive to many chemotherapeutic agents [18]. As demonstrated by LD₅₀ determination and biochemical data, co-treatment with PARP inhibitors sensitized EVSA-T and MDA-MB-231 cells to doxo-induced apoptosis. Significant increases in the proteolysis of cell death substrates and DNA fragmentation (sub G₁) further verified a caspase 3-mediated sensitization in doxo-induced apoptosis.

Doxo is an active chemotherapeutic agents used in clinical oncology. Doxo is a key adjuvant drug for breast cancer treatment. It triggers apoptosis through several mechanisms. As with many chemotherapeutic agents, it induces DNA damage by interacting with topoisomerase II, leading to DNA breakage [19]. So far, there are no reports describing PARP activation by topoisomerase II inhibitors. In the current study we have found a rapid activation of PAR synthesis following doxo treatment, suggesting a direct effect of doxo on PARP activity (figure 1).

The ability of PARP inhibitors to potentiate drug-induced cell death in tumour cells has been shown in multiple studies due to their potential application as chemo and radio-potentiators [7],[20]. Although there are examples showing direct toxic effects of PARP inhibitors in tumour cells [21] most of the studies focus on the potentiating effects of PARP inhibitors on alkylating agents or ionising radiation-induced tumour cell death.

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The exposure of cells to ionising radiation leads to hydroxyl radical mediated DNA injury whereas alkylating agents directly damage DNA. Other types of cytotoxic drugs such as topoisomerase I and II inhibitors may also lead to DNA breakage. A previous study has shown that the DNA strand breaks induced by the topoisomerase I inhibitor, camptothecin, were increased by the PARP inhibitor NU1025 and exposure to camptothecin-activated PARP. In contrast, NU1025 did not increase the DNA strand breakage or cytotoxicity caused by the topoisomerase II inhibitor etoposide [22]. However, in our model we have found that PARP is involved in the cellular response to doxo-mediated DNA damage. This is probably the first report showing that PARP inhibition increases the cytotoxic effects by topoisomerase II inhibitor.

Because of this, deficient repair of DNA breaks following inhibition of PARP leads to accumulation of DNA damage and shift from a repair response to an apoptotic one. This apoptotic response is in addition p53-independent, since EVSA-T and MDA-MB-231 contains a mutant-inactive p53. The mechanism by which ANI facilitates doxo-induced apoptosis is not related to a decreased NF- κ B response (figure 4) but rather to an acceleration of apoptosis due to an increased loss in mitochondrial potential leading to the activation of the final caspase-3, as revealed by the increase in PARP-1 cleavage and oligonucleosomal DNA fragmentation (sub-G₁, figure 2C and 2D). Moreover, the CFA assay shows a striking potentiation of doxo-induced cell death following co-treatment with ANI, suggesting that long-term effect of doxo is amplified with the use of PARP inhibitors, minimizing clonal expansion of resistant tumour cells. A recent report has also described that the use of PARP inhibitors together with doxo reduces doxo-induced cardiac dysfunction by avoiding necrotic cell death [23].

On the basis of these data, PARP inhibitors may be potentially useful in combination with topoisomerase II inhibitors anticancer chemotherapy in p53 deficient tumours, which is the direct cause of resistance to chemotherapy .

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Figure legends

Figure 1. PARP activation after doxo treatment.

(A) Time course of PAR formation in i) EVSA-T, ii) MDA-MB-231 cell lines. After treatment with doxo 1 μ g/ml (1 hour), cells were harvested and analyzed by immunoblotting for poly(ADP-ribose) formation. PARP-1 was used as loading control. **(B)** Immunofluorescence staining of EVSA-T cells for poly(ADP-ribose) (PAR) formation (green) and nuclei are counterstained with propidium iodide (red): i) control, ii) ANI 10 μ M, iii) treated with doxo 1 μ g/ml, 1 hour, iv) EVSA-T treated with doxo 1 μ g/ml + ANI 10 μ M. Overlaid images show that doxo induces PAR formation in nuclei (yellow), this effect is reduced with ANI treatment. Cells grown on cover-slips were fixed 40 minutes after treatment with doxo.

Figure 2. Effects of PARP inhibition on cell death after doxo treatment

(A) Cell viability in EVSA-T cell line was assessed by the sulphorhodamine B assay in the presence of increasing concentrations of doxo until 72 hours; results are the average of three separate experiments. **(B)** Induction of apoptosis 24 hours after treatment with 1 μ g/ml doxo in EVSA-T cell line, measured by PS externalization (Annexin-V) using flow cytometry analysis. The PARP inhibitor ANI (10 μ M) increased substantially cell death induced by doxo (2.25 x). Analysis of doxo-induced cell death with or without PARP inhibitor (ANI 10 μ M) and pan-caspase inhibitor (Z-VAD.fmk, 50 μ M) showed that cell death induced by doxo was caspase-dependent. Cytotoxicity of PARP inhibitor (ANI 10 μ M) was measured and showed a low toxicity (4%) 24 hours after treatment. Error bars represent SE of the mean (SEM) from at least four independent experiments. * $p < 0.05$ respect to control cells, cells treated with ANI and doxo + ANI treated cells. ** $p < 0.001$ respect to control cells, cells treated with ANI. **(C, D)** Cell death 48 hours after 1 μ g/ml doxo treatment in EVSA-T (C) and MDA-MB-231 (D) cell lines, measured by PI staining and flow cytometry analysis. The cytotoxic effect of doxo is increased by PARP

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inhibition 1.7 times in EVSA-T and 2 times in MDA-MB-231. Cytotoxicity of PARP inhibitor (ANI 10 μ M) was measured and showed a low toxicity (6% EVSA-T and 2.9% in MDA-MB-231) 48 hours after treatment. Error bars represent SE of the mean (SEM) from at least four independent experiment. * $p < 0.001$ respect to control cells and cells treated with ANI. ** $p < 0.001$ respect to cells treated with doxo. **(E)** Colony forming assay (CFA) after incubation with different concentrations of doxo and with or without PARP inhibitor (ANI 10 μ M). ANI co-treatment significantly potentiated the cytotoxicity of doxo. CFA with ANI alone gave essentially the same result as the untreated control. Survival was determined from triplicates of three independent experiments and normalized for the plating efficiency or untreated controls. Error bars represent SE of the mean (SEM).

Figure 3. Effects of PARP inhibition on mitochondrial membrane potential and PARP-1 cleavage after doxo treatment.

(A) Mitochondrial membrane potential in EVSA-T cell line, 24 and 48 hours after treatment with 1 μ g/ml doxo, detected by DIOC6 staining and flow cytometry analysis. Marker indicates the region of cell population used for analysis. **(B,C)** Depolarization of mitochondrial membrane analysis in EVSA-T (B, 24 hours and C, 48 hours). Mitochondrial membrane depolarization triggered by doxo was increased after PARP inhibition (ANI 10 μ m) in both times. Error bars represent SE of the mean (SEM) from at least three independent experiments. * $p < 0.001$ respect to cells only with ANI and cells treated only with doxo **(D)**, Cytochrome c release from mitochondria to cytosolic fractions and translocation of cytosolic Bax to mitochondrial fractions following 1 μ g/ml doxo or doxo + ANI treatment in EVSA-T cells. **(E)** Caspase-mediated PARP-1 cleavage in EVSA-T was determined by Western blot. The 85 and 28 KDa fragment of PARP cleavage is shown 24 and 48 hours after of 1 μ g/ml doxo treatment. PARP-1 cleavage triggered by doxo was increased after PARP inhibition in both times.

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Figure 4. Doxo-induced activation of NF- κ B is not counteracted by ANI

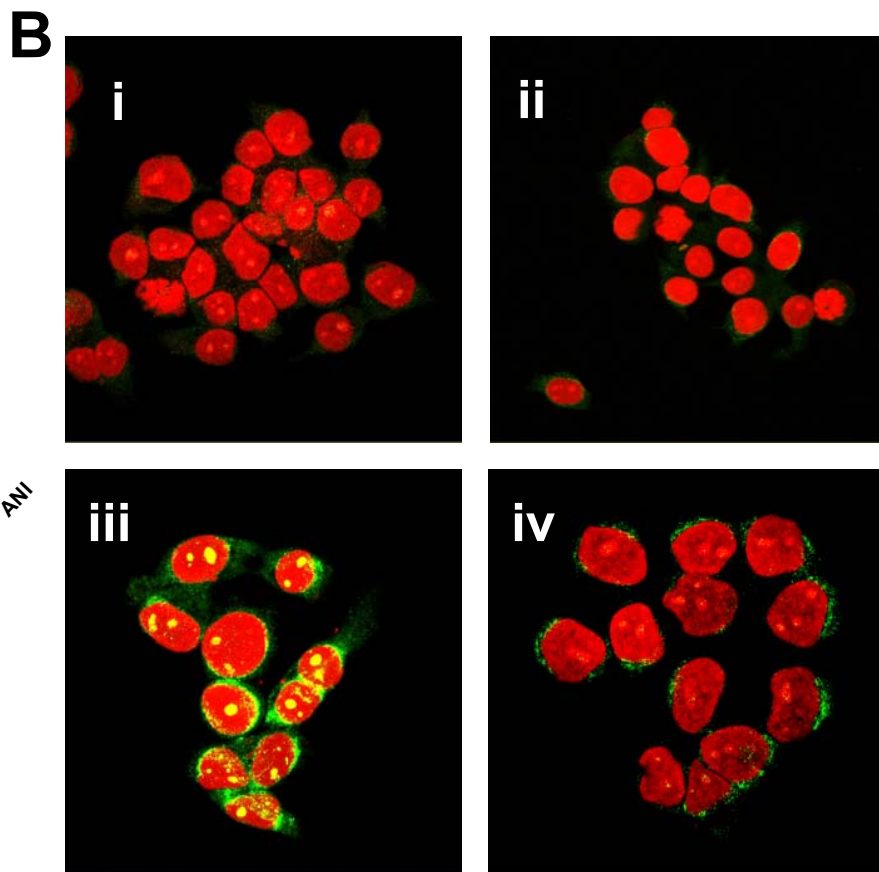
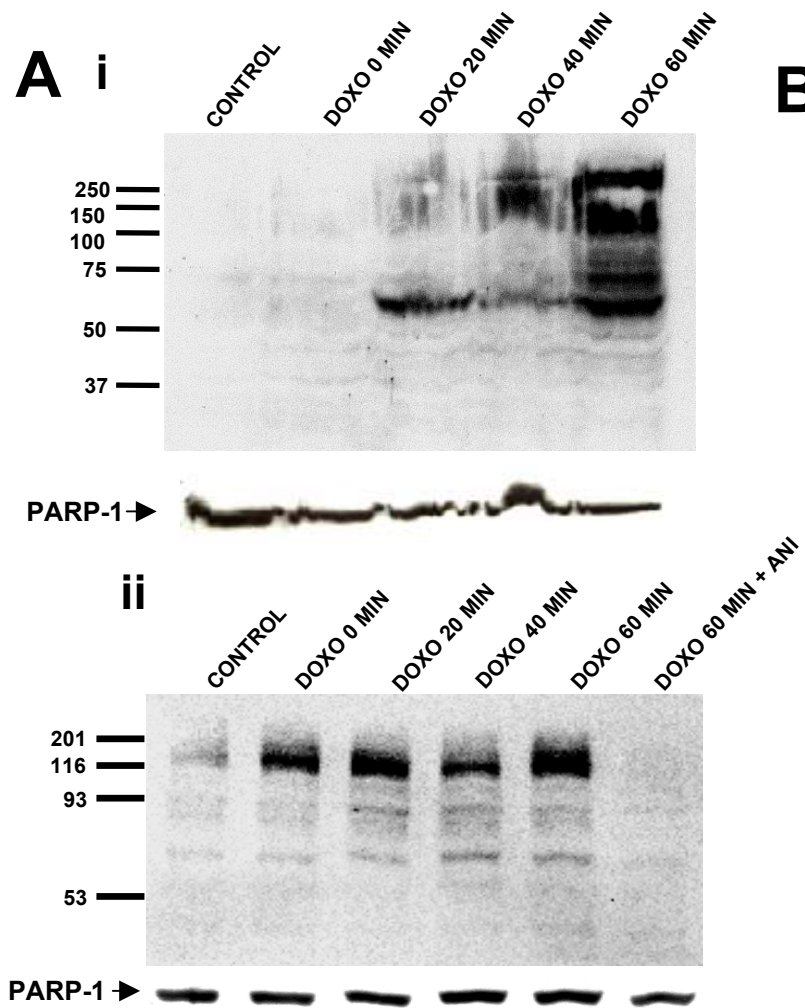
Band shift analysis of NF- κ B activation using κ B iNOS promoter sequence in different conditions in EVSA-T cell lines: untreated control, positive control NF- κ B activation TNF- α (20 ng/ml 2 hours), cells incubated with ANI (10 μ M), cells treated with doxo (1 μ g/ml) and cells with doxorubicin (1 μ g/ml) plus ANI (10 μ M), 24 hours after treatment. Competition with unlabelled probe was used to confirm that the shifted complex was NF- κ B. NF- κ B activation is similar in cells treated with doxo and cells with doxo+ANI. The results shown are representative of three independent experiments.

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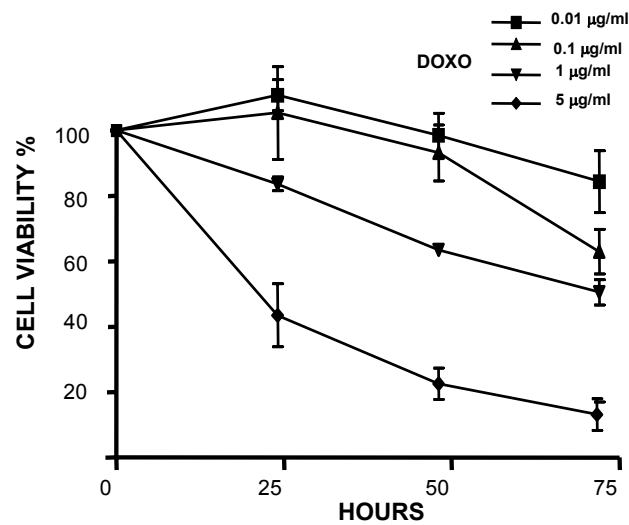
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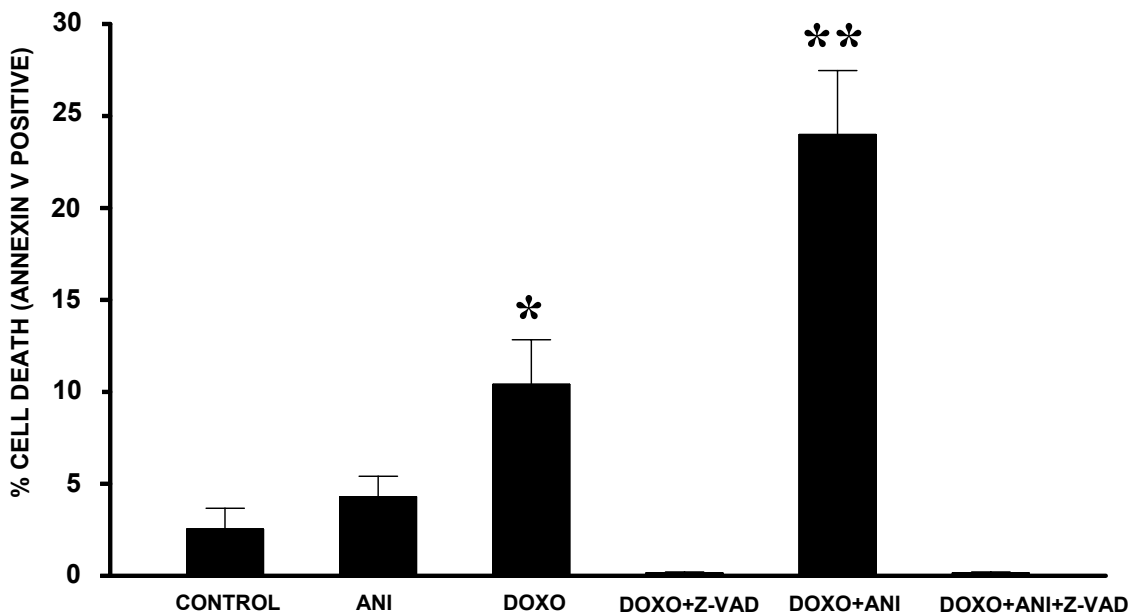
FIGURE 1



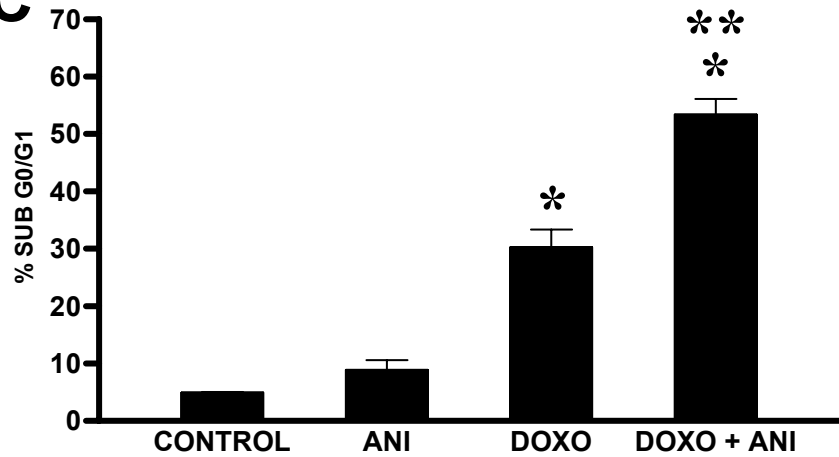
A *FIGURE 2*



B



C



D

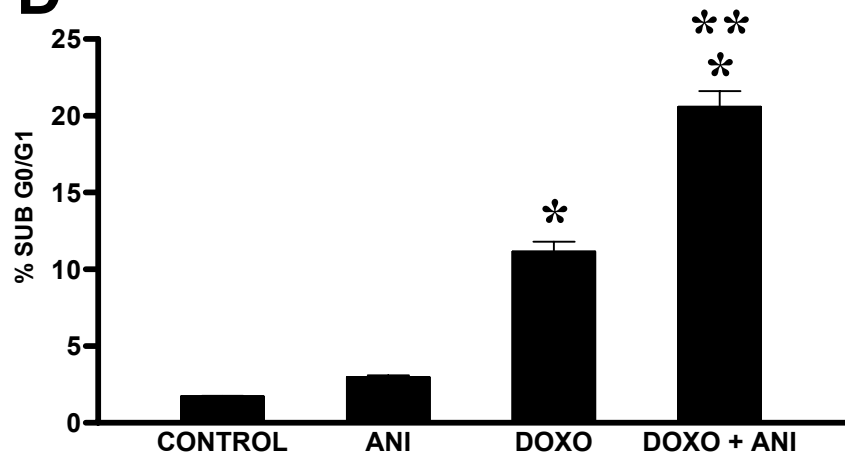


FIGURE 2

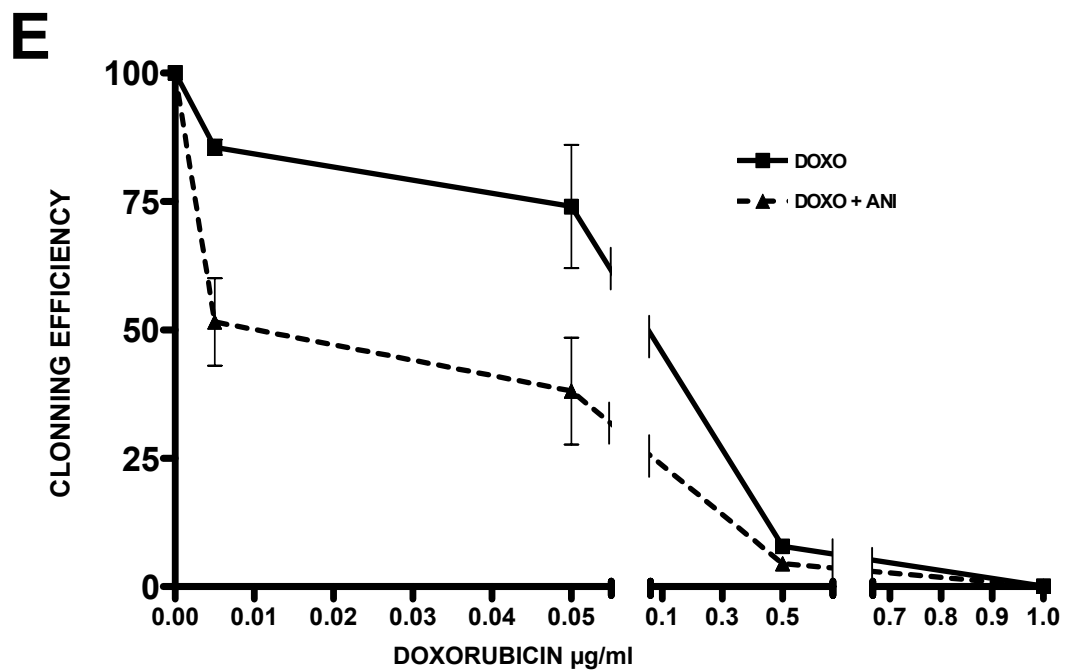
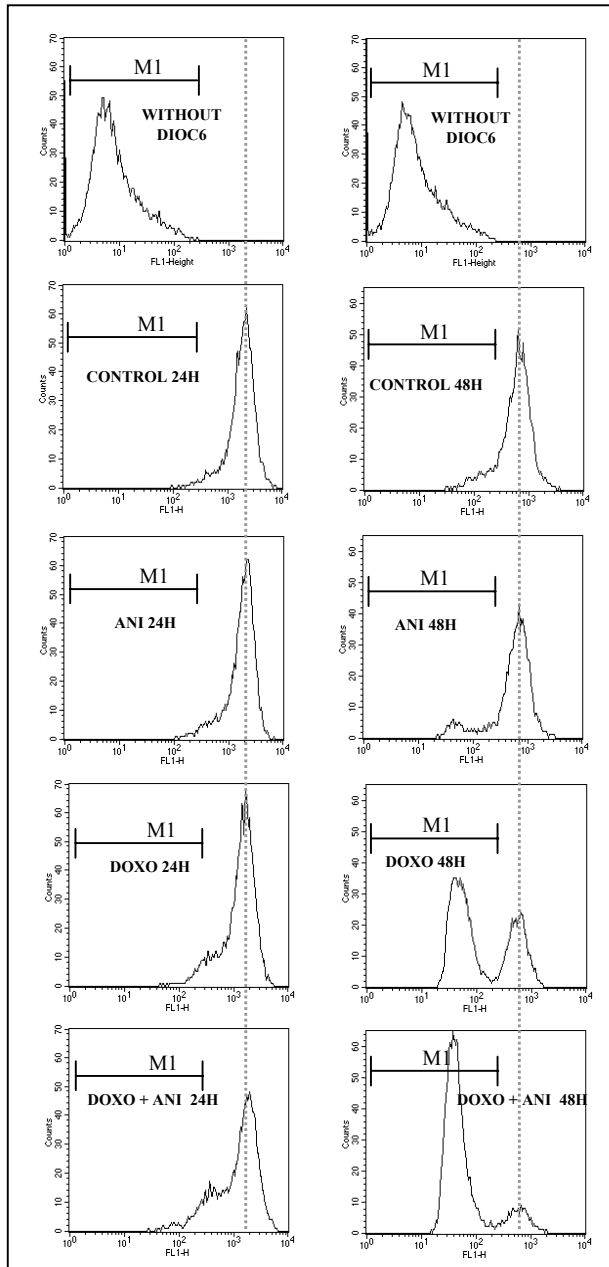
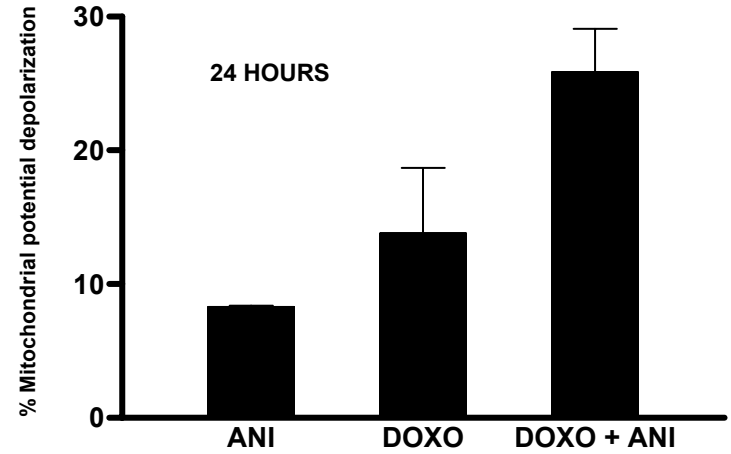


FIGURE 3

A



B



C

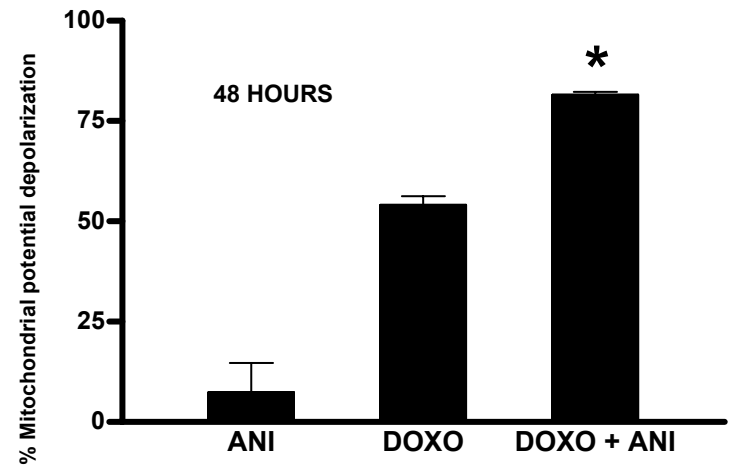
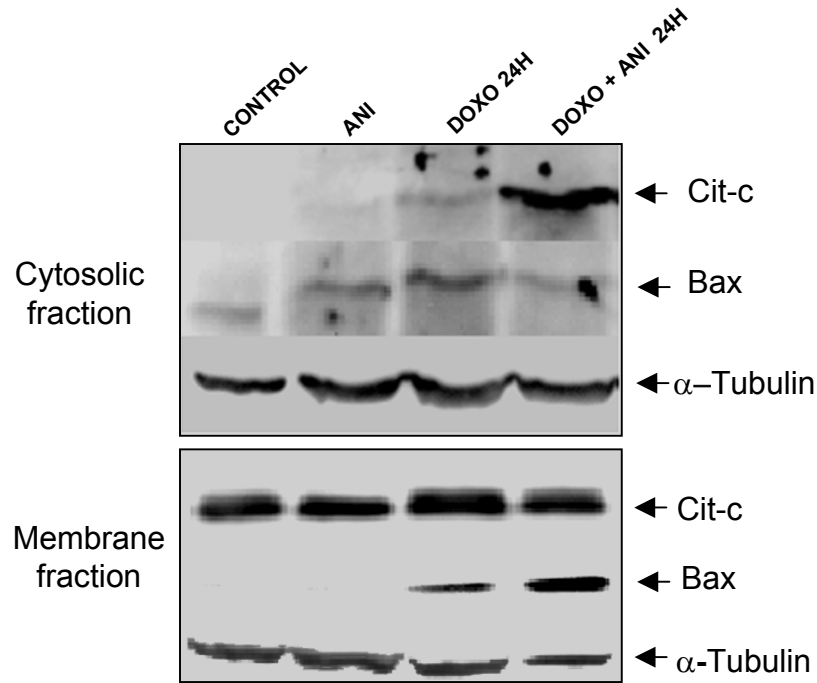


FIGURE 3

D



E

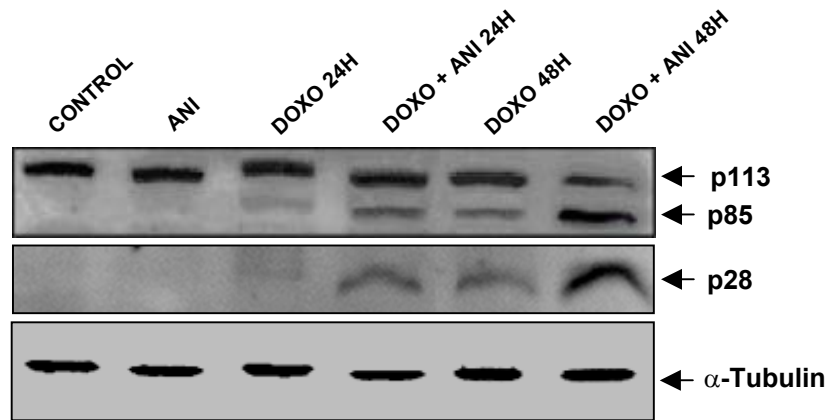


FIGURE 4

