PARP inhibition sensitizes p53-deficient breast cancer cells to doxorubicin-induced apoptosis

José Antonio MUÑOZ-GÁMEZ*, David MARTÍN-OLIVA*, Rocío AGUILAR-QUESADA†, Ana CAÑUELO‡, M. Isabel NUÑEZ§, M. Teresa VALENZUELA*, J. M. RUIZ DE ALMODÓVAR§, Gilbert DE MURCIA‖ and F. Javier OLIVER†[1]

*Hospital Universitario San Cecilio, Granada, Spain, †Instituto de Parasitologia y Biomedicina, CSIC, Avda. del Conocimiento s/n, 18100-Armilla, Granada, Spain, ‡Departamento de Biología Experimental, Universidad de Jaén, Jaén, Spain, §Departamento de Radiologia y Medicina Física, Universidad de Granada, Granada, Spain, and ‖UPR 9003 du CNRS, École de Supérieure de Biotechnologie de Strasbourg, Strasbourg, France

p53 deficiency confers resistance to doxo (doxorubicin), a clinically active and widely used antitumour anthracycline antibiotic. The purpose of the present study was to investigate the reversal mechanism of doxo resistance by the potent PARP [poly(ADP-ribose) polymerase] inhibitor ANI (4-amino-1,8-naphthalimide) 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 blotting and immunofluorescence of poly(ADP-ribose), and ANI enhanced the cytotoxic activity of doxo 2.3 times and in a caspase-dependent manner. The long-term cytotoxic effect was studied by a colony-forming assay. Using this assay, ANI also significantly potentiates the long-term cytotoxic effect with respect to treatment with doxo alone. Decrease in mitochondrial potential together with an increase in cytochrome *c* release, association of Bax with 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.

Key words: apoptosis, Bax, breast cancer, chemotherapy, doxorubicin, mitochondria, p53, poly(ADP-ribose) polymerase-1 (PARP-1).

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.

PARP-1 [poly(ADP-ribose) polymerase-1] is the principal member of a family of enzymes possessing poly(ADP-ribosylation) catalytic capacity. It 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 [7]. In particular, higher potency PARP inhibitors have a greatly decreased effect on mono(ADP-ribosyl) transferase enzymes. ANI (4-amino-1,8-naphthalimide) 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.

The objectives of the present study were to investigate whether ANI could potentiate the cytotoxic effect of doxo (doxorubicin) in the 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 these cell lines. Our results show that doxo induces 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 results suggest that PARP inhibition may represent 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 Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum at 37 °C in a humidified 5 % CO_2 atmosphere. Cells were plated for 24 h before doxo treatment.

Abbreviations used: ANI, 4-amino-1,8-naphthalimide; CFA, colony-forming assay; doxo, doxorubicin; NF-κB, nuclear factor κB; PAR, poly(ADP-ribose); PARP, PAR polymerase; PI, propidium iodide; Z-Val-Ala-DL-Asp-CH_2F, benzyloxycarbonyl-valylalanyl-DL-aspartylfluoromethane.

[1] To whom correspondence should be addressed (email joliver@ipb.csic.es).

Drugs

Cells were treated with doxo for 1 h in Dulbecco's modified Eagle's medium supplemented with 10 % foetal bovine serum at 37 °C in a humidified 5 % CO_2 atmosphere. The PARP inhibitor ANI (10 μM) was dissolved in culture medium immediately before use. ANI solutions (10 μM) also contained < 2 % DMSO to improve solubility. ANI is sparingly soluble in water without adding DMSO. ANI was added 1 h before doxo treatment and thereafter present in the culture throughout the experiment. The pan-caspase inhibitor Z-Val-Ala-DL-Asp-CH_2F (benzyloxycarbonyl-valylalanyl-DL-aspartylfluoromethane, also known as Z-VAD-FMK; 50 μM) was added 2 h before doxo treatment and was thereafter present in the culture throughout the experiment.

Analysis of cell death

Cell viability was evaluated as described previously by the sulphorhodamine B method [11]. Measurement of apoptosis was determined by annexin V staining. After drug treatments, cells were harvested using trypsin-EDTA, washed once with ice-cold PBS and resuspended in 1 ml of annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Then, 75 000 cells were stained with 5 μl of annexin V FLUOS (Roche Molecular Biochemicals) in 100 μl of annexin V buffer at 4 °C. After 30 min, 100 μl of binding buffer was added to each tube and samples were analysed using a tri-laser FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

Sub-G_1 analysis was examined by flow cytometry using the PI (propidium iodide) DNA-staining method. Cells were harvested with trypsin-EDTA, washed once with ice-cold PBS and resuspended in 100 μl of PBS. Ice-cold ethanol (70 %, 900 μl) was added to the cells for 5 min, washed with 2 ml of PBS and the cells were resuspended in 250 μl of PI/RNase solution (PBS, 100 μg/ml RNase and 40 μg/ml PI). After 30 min, samples were analysed using a tri-laser FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson).

CFA (colony-forming assay)

Semi-confluent culture flasks were trypsinized, and adequate number of cells were seeded in 25 cm^2 tissue culture flasks. One day later, cells were exposed to doxo at the indicated concentrations for 1 h. Cells were treated with the PARP inhibitor ANI (10 μM) for 24 h. Cells were stained with crystal violet, 15 or 20 days later, 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 PAR [poly(ADP-ribose)] was performed on the cells, which were grown on glass coverslips and fixed in ice-cold methanol/acetone (1:1, v/v) for 10 min. PAR was detected by immunofluorescence using the monoclonal antibody 10H (Alexis, Grünberg, Germany) and FITC-conjugated goat anti-mouse immunoglobulins (Sigma, St. Louis, MO, U.S.A.). Nuclear counterstaining with PI was performed after removal of excess secondary antibody. Immunostaining was visualized with a Leica Spectral confocal laser microscope.

Western-blot analysis

Cells were detached from the culture flask, washed with PBS and resuspended in 100 μl of lysis buffer (50 mM Tris/HCl, pH 8, 0.1 mM EDTA, 0.5 % Triton X-100 and 12.5 mM 2-mercaptoethanol) for 30 min on ice. The pellet was eliminated and a sample buffer [50 mM Tris/HCl, pH 6.8, 6 M urea, 6 % 2-mercaptoethanol, 3 % (w/v) SDS and 0.003 % Bromophenol Blue] was added to the supernatant. Proteins were resolved by SDS/PAGE (12 % gel) and transferred on to Immun-Blot PVDF membranes (Bio-Rad). The blot was blocked with 5 % (w/v) milk powder in PBS containing 0.1 % Tween 20 for 30 min, washed with PBS/Tween, incubated overnight with the antibodies PAR (Biomol, Hamburg, Germany), anti-PARP-1 (EGF-69), anti-Bax and anti-cytochrome *c* (Pharmingen, San Diego, CA, U.S.A.) and anti-α-tubulin (Sigma) and incubated for 2 h with appropriate secondary antibodies. Bands were visualized by ECL-PLUS (Amersham Biosciences) and pictures were taken with the imaging system ChemiDoc XRS System (Bio-Rad).

For the measurements of cytochrome *c* and Bax, cells were washed with PBS and lysed for 5 min in 30 μl of ice-cold lysis buffer (80 mM KCl, 250 mM sucrose, 500 μg/ml digitonin, 1 μg/ml 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 ***g***. Proteins from the supernatant (cytosolic fraction) and pellet (membrane fraction) were mixed with sample buffer and resolved by SDS/PAGE (12 % gel). Cytochrome *c* and Bax were determined by Western-blot analysis as described above.

Detection of mitochondrial membrane potential

Cells were plated in 6-well plates (2×10^5 cells) and grown for 24 h. After this period, the cells were treated with doxo (1 μg/ml) and doxo (1 μg/ml) + ANI (10 μM). Cells were harvested using trypsin-EDTA, washed once with ice-cold PBS and resuspended in PBS with 40 nM $DIOC_6$ (3,3′-dihexyloxacarboxyanine iodide; Molecular Probes, Eugene, OR, U.S.A.) for 30 min at 37 °C. Samples were then analysed with an FACScan cytometer (Becton Dickinson) and the fluorescence was detected in FL1. During analysis of the flow-cytometric data, a marker indicating a cell population having lower $\Delta\psi_m$ was applied to histograms, and the percentage of cells in the region was determined. The number of cells with lower membrane potential (cells treated with doxo, with or without ANI) were expressed as a percentage of the cells without treatment (control cells).

Electrophoretic mobility-shift assay

The double-stranded oligonucleotides 5′-TGCTAGGGGGATTTTCCCTCTTCTTGT-3′ [12] with the sequences of the binding sites for NF-κB (nuclear factor κB) of the inducible nitric oxide synthase 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 μg of protein) were incubated with 2 μl of ^{32}P-labelled probe (6×10^4 d.p.m.) in a final volume of 20 μl 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.

RESULTS

Doxo treatment induces PARP activation

Many different cell insults infringing DNA damage have been shown to be capable of activating PARP. Doxo is a powerful DNA-damaging agent but there is no evidence 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 demonstrate that

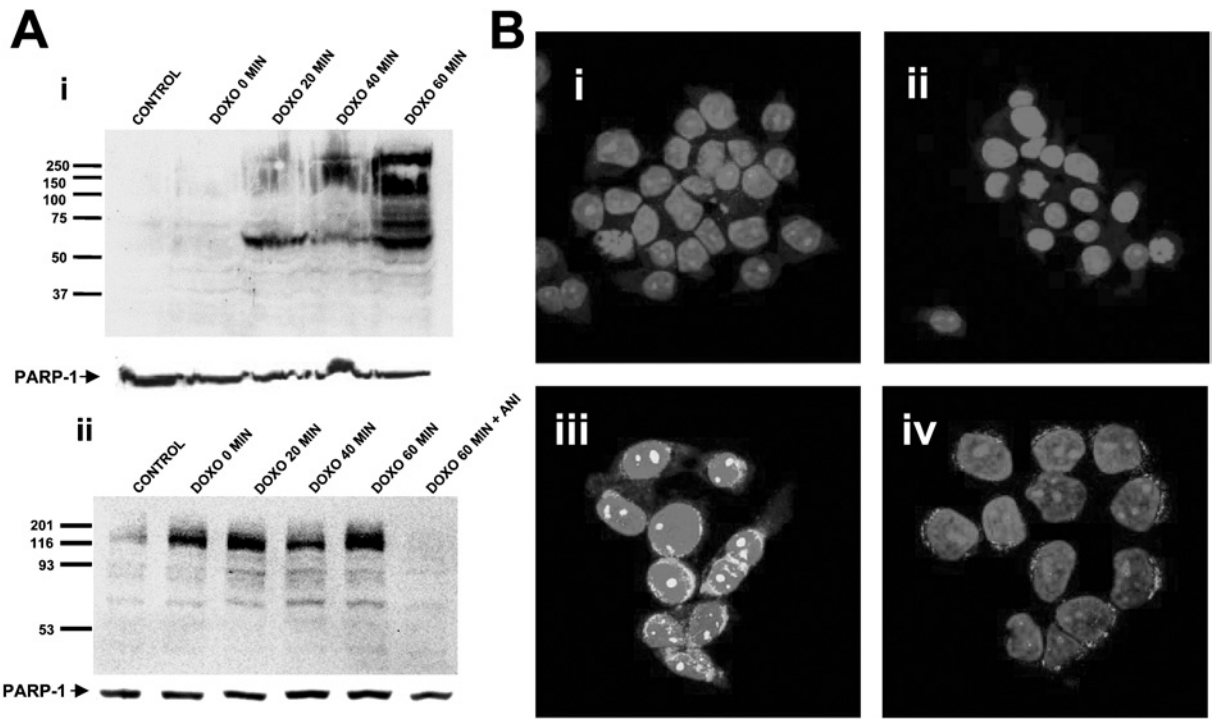

Figure 1 PARP activation after doxo treatment

(**A**) Time course of PAR formation in (i) EVSA-T and (ii) MDA-MB-231 cell lines. After treatment with 1 μg/ml doxo (1 h), cells were harvested and analysed by immunoblotting for PAR formation. PARP-1 was used as the loading control. (**B**) Immunofluorescence staining of EVSA-T cells for PAR formation. Nuclei are counterstained with PI: (i) control, (ii) 10 μM ANI, (iii) treated with doxo (1 μg/ml, 1 h), (iv) EVSA-T treated with doxo (1 μg/ml) + ANI (10 μM). Overlaid images show that doxo induces PAR formation in nuclei (white), and it is reduced with ANI treatment. Cells grown on coverslips were fixed for 40 min after treatment with doxo.

doxo induced PARP activation. In Figure 1, we show that doxo (1 μg/ml) is capable of inducing a rapid PARP activation (20 min after treatment) measured by Western blotting [Figure 1A(i) (EVSA-T) and 1A(ii) (MDA-MB-231)] or immunofluorescence [Figure 1B(iii), EVSA-T cells]. Pretreatment with 10 μM ANI completely prevented doxo-induced PARP activation [Figure 1B(iv)]. The action 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 performed a dose–response of doxo-induced cell death to determine at which dose these cells start to be sensitive to the drug. In short-term experiments of cell viability (until 72 h), they were completely resistant to doses below 1 μg/ml and partially sensitive to a dose of 1 μg/ml, reaching a 50 % 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 shown previously [11]. Then, we used annexin V and sub-G1 to evaluate cell death as a measure of short-term cytotoxic effects and CFA as a measure of long-term cytotoxic effects, and we also studied the effect of ANI co-treatment in a second p53-deficient breast cancer cell line, MDA-MD-231, to substantiate further our observation. Pretreatment with 10 μM ANI resulted in a potentiation of doxo-induced cell death measured by all three criteria [Figures 2B (annexin V in EVSA-T), 2C (sub-G_1 in EVSA-T), 2D (sub-G_1 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 (Figure 2C) or 2-fold (Figure 2D) with sub-G_1. Long-term cytotoxicity using ANI and doxo was also potentiated subsequent to treatment with doxo alone according to the CFA (Figure 2E). The effect of ANI was completely abolished with the pan-caspase inhibitor Z-Val-Ala-DL-Asp-CH_2F (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 analyse more precisely 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 the hallmark of doxo-induced apoptosis) in EVSA-T cells. In Figures 3(A)–3(C), the change in mitochondrial permeability was poorly shifted by doxo alone, and the pretreatment with ANI further increased the decrease 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 capable of restoring a p53-like response in these cells.

Another mechanism by which tumour cells may be resistant to chemotherapy is by activation of the transcription factor NF-κB, 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 the response of NF-κB [13,14]. We assessed the impact of ANI on doxo-induced NF-κB activation using an electrophoretic mobility-shift assay and found that ANI does not affect the ability of

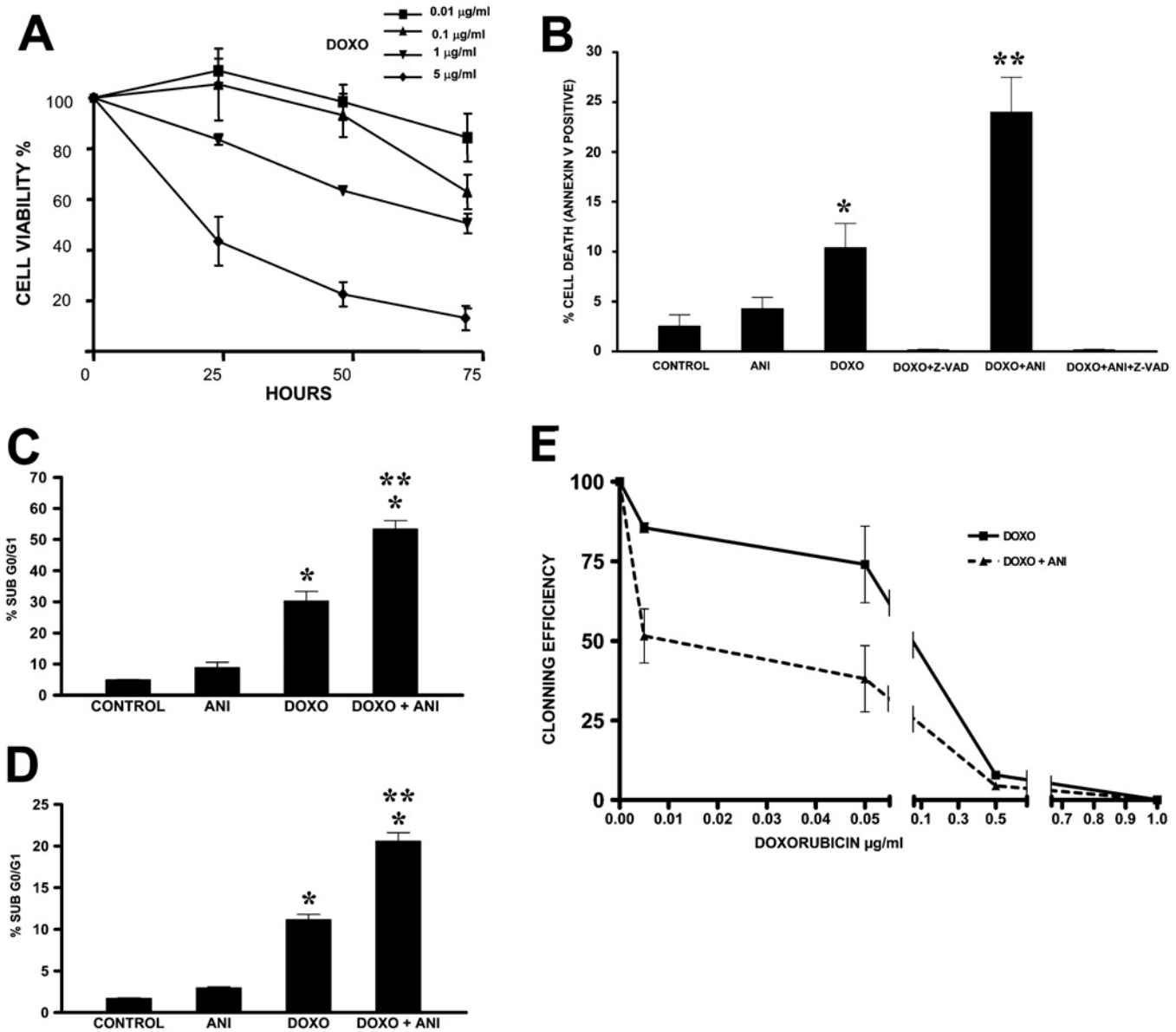

Figure 2 Effects of PARP inhibition on cell death after doxo treatment

(**A**) Cell viability in the EVSA-T cell line was assessed by the sulphorhodamine B assay in the presence of increasing concentrations of doxo until 72 h; results are the average of three separate experiments. (**B**) Induction of apoptosis, 24 h after treatment with 1 μg/ml doxo in the EVSA-T cell line, measured by phosphatidylserine externalization (annexin-V) using flow-cytometry analysis. The PARP inhibitor ANI (10 μM) significantly increased (2.25 times) the cell death induced by doxo. Analysis of doxo-induced cell death with or without the PARP inhibitor (ANI, 10 μm) and the pan-caspase inhibitor Z-Val-Ala-DL-Asp-CH_2F (Z-VAD; 50 μM) showed that the cell death induced by doxo was caspase-dependent. Cytotoxicity of the PARP inhibitor (ANI, 10 μM) was measured and it showed a low toxicity (4 %) 24 h after treatment. Error bars represent the S.E.M. for at least four independent experiments. *$P < 0.05$ compared with control cells, cells treated with ANI and cells treated with doxo + ANI. **$P < 0.001$ compared with control and ANI-treated cells. (**C**, **D**) Cell death 48 h after treatment with 1 μg/ml doxo in EVSA-T (**C**) and MDA-MB-231 (**D**) cell lines, measured by PI staining and flow-cytometry analysis. The cytotoxic effect of doxo was increased by PARP inhibition 1.7 times in EVSA-T and two times in MDA-MB-231 cells. Cytotoxicity of the PARP inhibitor (ANI, 10 μM) was measured and it showed a low toxicity (6 % in EVSA-T and 2.9 % in MDA-MB-231) 48 h after treatment. Error bars represent the S.E.M. for at least four independent experiments. *$P < 0.001$ compared with control and ANI-treated cells. **$P < 0.001$ compared with cells treated with doxo. (**E**) CFA after incubation with different concentrations of doxo and with or without PARP inhibitor (ANI, 10 μM). 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 triplicate measurements from three independent experiments and normalized for the plating efficiency or untreated controls. Error bars represent S.E.M.

doxo to induce NF-κB activation, suggesting that ANI-induced potentiation of cell death is independent of NF-κB (Figure 4).

DISCUSSION

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

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 LD_{50} (median lethal dose) determination and biochemical data, co-treatment with

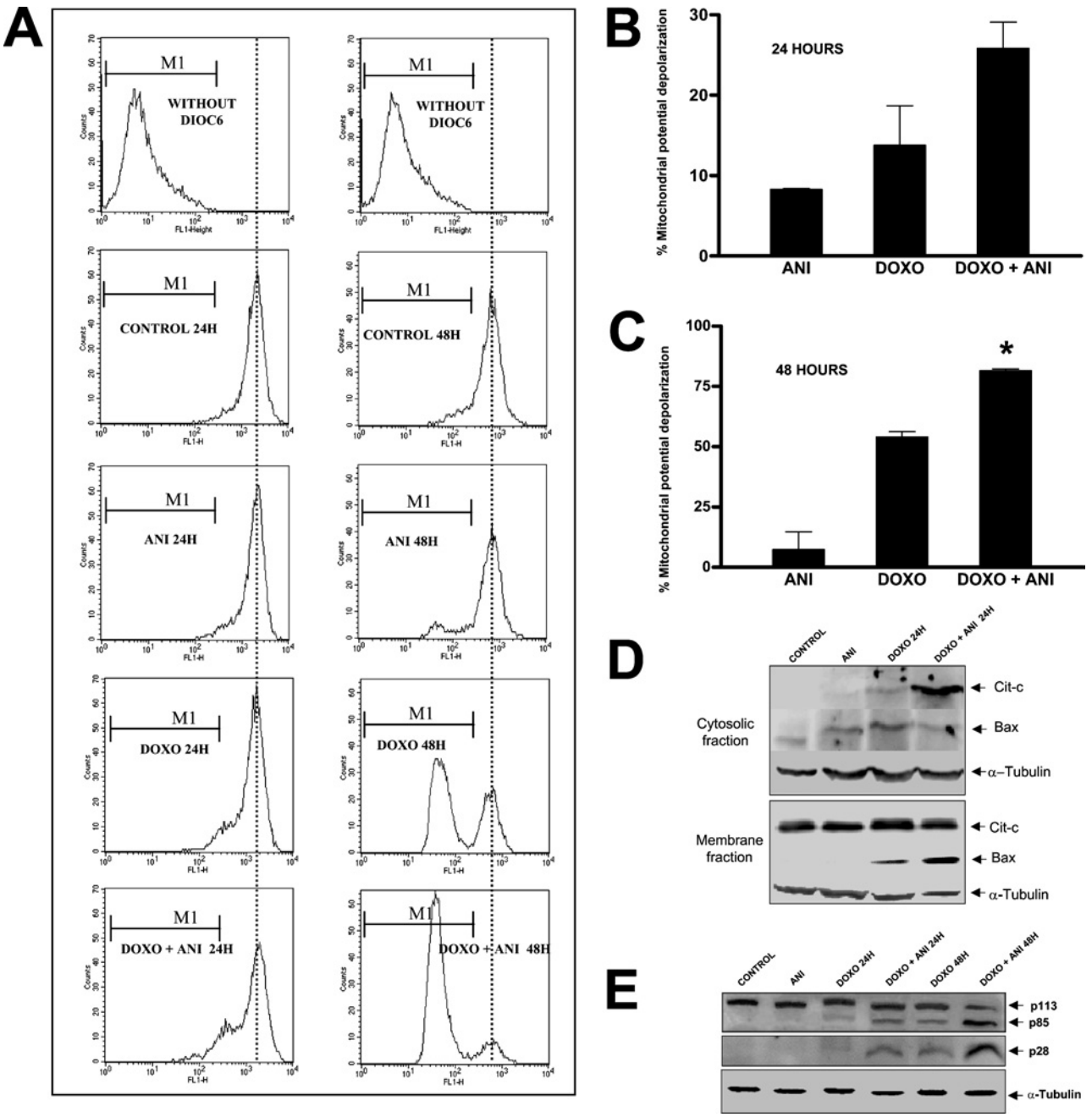

Figure 3 Effects of PARP inhibition on mitochondrial membrane potential and PARP-1 cleavage after doxo treatment

(**A**) Mitochondrial membrane potential in EVSA-T cell line, 24 and 48 h after treatment with 1 μg/ml doxo, detected by $DIOC_6$ staining and flow-cytometry analysis. Marker indicates the region of cell population used for analysis. (**B**, **C**) Analysis of mitochondrial membrane depolarization in EVSA-T for 24 h (**B**) and 48 h (**C**). Mitochondrial membrane depolarization triggered by doxo was increased after PARP inhibition (ANI, 10 μm) for both times. Error bars represent S.E.M. for at least three independent experiments. $^*P < 0.001$ compare with cells treated 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 after treatment with 1 μg/ml doxo or doxo + ANI in EVSA-T cells. (**E**) Caspase-mediated PARP-1 cleavage in EVSA-T was determined by Western blotting. The 85 and 28 kDa fragment of PARP cleavage is shown 24 and 48 h after 1 μg/ml doxo treatment. PARP-1 cleavage triggered by doxo was increased after PARP inhibition for both times.

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-G_1) further verified a caspase 3-mediated sensitization in doxo-induced apoptosis.

Doxo is an active chemotherapeutic agent 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 present study, we have found a rapid activation of PAR synthesis after 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

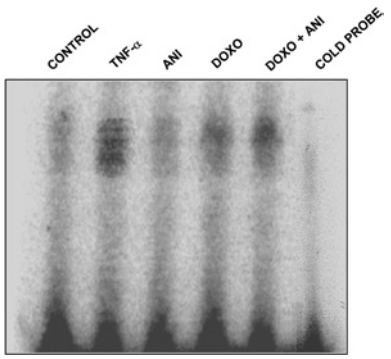

Figure 4 The doxo-induced activation of NF-κB is not counteracted by ANI

Band-shift analysis of NF-κB activation using the κB inducible nitric oxide synthase promoter sequence under different conditions in EVSA-T cell lines: untreated control, internal control for NF-κB activation using TNF-α (20 ng/ml, 2 h), cells incubated with ANI (10 μM), cells treated with doxo (1 μg/ml) and cells treated with doxo (1 μg/ml) + ANI (10 μM), 24 h after treatment. Competition with an unlabelled probe was used to confirm that the shifted complex was NF-κB. NF-κB activation is similar in cells treated with doxo and cells treated with doxo + ANI. The results shown are representative of three independent experiments.

their potential application as chemo- and radiopotentiators [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 ionizing radiation-induced tumour cell death.

Exposure of cells to ionizing 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 on 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.

Owing to this, deficient repair of DNA breaks after the inhibition of PARP leads to accumulation of DNA damage and shift from a repair response to an apoptotic one. This apoptotic response is, moreover, p53-independent, since EVSA-T and MDA-MB-231 contain a mutant-inactive p53. The mechanism by which ANI facilitates doxo-induced apoptosis is related not to a decreased NF-κB response (Figure 4) but rather to an acceleration of apoptosis due to an increased loss of the mitochondrial potential, leading to activation of the final caspase 3, as revealed by the increase in PARP-1 cleavage and oligonucleosomal DNA fragmentation (sub-G_1, Figures 2C and 2D). Moreover, the CFA assay shows a striking potentiation of doxo-induced cell death after co-treatment with ANI, suggesting that the long-term effect of doxo is amplified with the use of PARP inhibitors, minimizing clonal expansion of resistant tumour cells. A recent work has also reported 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 results, PARP inhibitors may be potentially useful, in combination with topoisomerase II inhibitors, in anticancer chemotherapy in p53-deficient tumours, which is the direct cause of resistance against chemotherapy.

We acknowledge Dr A. Lopez-Rivas (Consejo Superior de Investigaciones Cientificas, Granada, Spain) for a helpful discussion. This work was supported by grants FIS 00/0948, FIS G03/152 and SAF:2003-01217 to F.J.O., SAF:2001-3533 to J.M.R.A. and FIS CP03/00142 to M.T.V., J.A.M.G. and D.M.O. are recipients of fellowships from FIS, and R.A.Q. from Ministerio de Educación y Ciencia.

REFERENCES

1 Johnstone, R. W., Ruefli, A. A. and Lowe, S. W. (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell (Cambridge, Mass.) **108**, 153–164
2 Shin, M. S., Kim, H. S., Lee, S. H., Park, W. S., Kim, S. Y., Park, J. Y., Lee, J. H., Lee, S. K., Lee, S. N., Jung, S. S. et al. (2001) Mutations of tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2) genes in metastatic breast cancers. Cancer Res. **61**, 4942–4946
3 Geisler, S., Lonning, P. E., Aas, T., Johnsen, H., Fluge, O., Haugen, D. F., Lillehaug, J. R., Akslen, L. A. and Borresen-Dale, A. L. (2001) Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer: prognostic significance of apoptosis regulators in breast cancer. Cancer Res. **61**, 2505–2512
4 Krajewski, S., Krajewska, M., Turner, B. C., Pratt, C., Howard, B., Zapata, J. M., Frenkel, V., Robertson, S., Ionov, Y., Yamamoto, H. et al. (1999) Prognostic significance of apoptosis regulators in breast cancer. Endocr. Relat. Cancer **6**, 29–40
5 Makin, G. and Dive, C. (2001) Apoptosis and cancer chemotherapy. Trends Cell Biol. **11**, S22–S26
6 Shall, S. and de Murcia, G. (2000) Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? Mutat. Res. **460**, 1–15
7 Miknyoczki, S. J., Jones-Bolin, S., Pritchard, S., Hunter, K., Zhao, H., Wan, W., Ator, M., Bihovsky, R., Hudkins, R., Chatterjee, S., Klein-Szanto, A. et al. (2003) Chemopotentiation of temozolomide, irinotecan, and cisplatin activity by CEP-6800, a poly(ADP-ribose) polymerase inhibitor. Mol. Cancer Ther. **2**, 371–382
8 Schlicker, A., Peschke, P., Burkle, A., Hahn, E. W. and Kim, J. H. (1999) 4-Amino-1,8-naphthalimide: a novel inhibitor of poly(ADP-ribose) polymerase and radiation sensitizer. Int. J. Radiat. Biol. **75**, 91–100
9 Ruiz-Ruiz, M. C. and Lopez-Rivas, A. (1999) p53-mediated up-regulation of CD95 is not involved in genotoxic drug-induced apoptosis of human breast tumor cells. Cell Death. Differ. **6**, 271–280
10 Ostrakhovitch, E. A. and Cherian, M. G. (2004) Differential regulation of signal transduction pathways in wild type and mutated p53 breast cancer epithelial cells by copper and zinc. Arch. Biochem. Biophys. **423**, 351–361
11 Valenzuela, M. T., Nunez, M. I., Villalobos, M., Siles, E., Olea, N., Pedraza, V., McMillan, T. J. and Ruiz de Almodovar, J. M. (1995) Relationship between doxorubicin cell sensitivity, drug-induced DNA double-strand breaks, glutathione content and P-glycoprotein in mammalian tumor cells. Anti-Cancer Drugs **6**, 749–757
12 Xie, Q. W., Kashiwabara, Y. and Nathan, C. (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J. Biol. Chem. **269**, 4705–4708
13 Martin-Oliva, D., O'Valle, F., Munoz-Gamez, J. A., Valenzuela, M. T., Nunez, M. I., Aguilar, M., Almodovar, J. R., Moral, R. G. and Oliver, F. J. (2004) Crosstalk between PARP-1 and NF-kappaB modulates the promotion of skin neoplasia. Oncogene **23**, 5275–5283
14 Oliver, F. J., Menissier-de Murcia, J., Nacci, C., Decker, P., Andriantsitohaina, R., Muller, S., de la Rubia, G., Stoclet, J. C. and de Murcia, G. (1999) Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly (ADP-ribose) polymerase-1 deficient mice. EMBO J. **18**, 4446–4454
15 Banasik, M., Komura, H., Shimoyama, M. and Ueda, K. (1992) Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase. J. Biol. Chem. **267**, 1569–1575
16 Lin, A. and Karin, M. (2003) NF-kappaB in cancer: a marked target. Semin. Cancer Biol. **13**, 107–114
17 Pratt, M. A., Bishop, T. E., White, D., Yasvinski, G., Menard, M., Niu, M. Y. and Clarke, R. (2003) Estrogen withdrawal-induced NF-kappaB activity and bcl-3 expression in breast cancer cells: roles in growth and hormone independence. Mol. Cell. Biol. **23**, 6887–6900
18 de Almodovar, C. R., Ruiz-Ruiz, C., Rodriguez, A., Ortiz-Ferron, G., Redondo, J. M. and Lopez-Rivas, A. (2004) Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) decoy receptor TRAIL-R3 is up-regulated by p53 in breast tumor cells through a mechanism involving an intronic p53-binding site. J. Biol. Chem. **279**, 4093–4101
19 Gudkov, A. V., Zelnick, C. R., Kazarov, A. R., Thimmapaya, R., Suttle, D. P., Beck, W. T. and Roninson, I. B. (1993) Isolation of genetic suppressor elements, inducing resistance to topoisomerase II-interactive cytotoxic drugs, from human topoisomerase II cDNA. Proc. Natl. Acad. Sci. U.S.A. **90**, 3231–3235

20 Virag, L. and Szabo, C. (2002) The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacol. Rev. **54**, 375–429
21 Mendeleyev, J., Kirsten, E., Hakam, A., Buki, K. G. and Kun, E. (1995) Potential chemotherapeutic activity of 4-iodo-3-nitrobenzamide. Metabolic reduction to the 3-nitroso derivative and induction of cell death in tumor cells in culture. Biochem. Pharmacol. **50**, 705–714
22 Bowman, K. J., Newell, D. R., Calvert, A. H. and Curtin, N. J. (2001) Differential effects of the poly (ADP-ribose) polymerase (PARP) inhibitor NU1025 on topoisomerase I and II inhibitor cytotoxicity in L1210 cells *in vitro*. Br. J. Cancer **84**, 106–112
23 Pacher, P., Liaudet, L., Bai, P., Virag, L., Mabley, J. G., Hasko, G. and Szabo, C. (2002) Activation of poly(ADP-ribose) polymerase contributes to development of doxorubicin-induced heart failure. J. Pharmacol. Exp. Ther. **300**, 862–867

Received 11 May 2004/7 September 2004; accepted 29 September 2004
Published as BJ Immediate Publication 29 September 2004, DOI 10.1042/BJ20040776