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A P-glycoprotein- and MRP1-independent Doxorubicin-resistant Variant of the MCF-7 Breast Cancer Cell Line with Defects in Caspase-6, -7, -8, -9 and -10 Activation Pathways

SOO-JUNG PARK, CHING-HAUNG WU and AHMAD R. SAFA

Department of Pharmacology and Toxicology and Indiana University Cancer Center, 1044 West Walnut R4-119, Indianapolis, IN 46202, U.S.A.

Abstract. *Background: Several mechanisms are known to cause resistance to chemotherapy in cancer cells, but the mechanisms of drug resistance due to a lack of apoptosis are not well elucidated. Materials and Methods: To understand the mechanisms of resistance to apoptosis induced by doxorubicin (DOX), we developed a DOX-resistant variant of MCF-7 referred to as MCF-7/Adr-20, measured growth inhibition by methylene blue cell survival assay, quantitated apoptosis by annexin V binding assay and detected activation of caspases-6, -7, -8, -9 and -10 in these cells. Results: The resistant cells expressed 20-fold resistance to apoptosis induced by DOX compared to MCF-7 cells. MCF-7/Adr-20 cells did not express MDR1 mRNA or its product P-glycoprotein and they did not overexpress MRP-1. Treating MCF-7 cells with 0.01, 0.1 and 1 μM DOX for 72 h induced 8, 14 and 28% apoptosis, respectively. However, only 1 μM DOX was able to trigger about 8% apoptosis in MCF-7/Adr-20 cells. Moreover, apoptosis triggered by 0.01 and 0.1 μM DOX in MCF-7 cells was mainly caspase-dependent, but at 1 μM about 70% of apoptosis was caspase-dependent. Western blot analysis revealed that caspase-7 was activated at 0.1 and 1 μM DOX treatment and caspases-6, -8, -9 and 10 were only activated at 1 μM DOX treatment in MCF-7 cells, but none of the caspases checked were activated in MCF-7/Adr-20 cells. Moreover, DOX at 0.01 and 0.1 μM induced p53 and $p21^{WAF1/CIP-1}$ to the same extent in both MCF-7 and MCF-7/Adr-20 cells. Therefore, while DOX triggers growth arrest and induces p53 and $p21^{WAF-1/CIP-1}$ in these cells, defects in activation of the initiator and executioner caspases play a major role in resistance to apoptosis triggered by DOX.*

Correspondence to: Ahmad R. Safa, Department of Pharmacology and Toxicology and Indiana University Cancer Center, 1044 West Walnut R4-119, Indianapolis, IN 46202, U.S.A. Tel: (317) 278-4952, Fax: (317) 274-8046, e-mail: asafa@iupui.edu

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Chemotherapeutic drug resistance is a major clinical problem and an important cause of treatment failure in cancer (1-4). Several mechanisms have been found that cause resistance to chemotherapeutic agents in cancer cells growing in culture. However, the significance of many of these mechanisms in clinical drug resistance remains to be found. The major chemotherapeutic drug resistance mechanisms (1-7) include: (a) overexpression of P-glycoprotein (P-gp), other plasma membrane multidrug transporters (*e.g.*, MRP1) and the breast cancer resistance protein (BCRP), each of which decreases the intracellular accumulation of chemotherapeutic agents; (b) overexpression of the lung resistance protein (LRP), a major vault protein with the possible function of reducing drug accumulation in the nucleus; (c) increased detoxification (*e.g.*, overexpression of glutathione and glutathione S-transferase π, multiorganic anionic transporter [MOAT]); (d) failure to undergo apoptosis caused by alterations in bcl-2, bcl_x or bax expression and up-regulation of inhibitor of apoptosis proteins (IAPs); (e) changes in the molecules involved in DNA repair (*e.g.*, methylguanine-DNA methyltransferase, human mismatch repair proteins and the CDK inhibitor $p21^{WAF1/C1P1}$); (f) alterations in drug targets (*e.g.*, topoisomerase I and II, dihydrofolate reductase); (g) activation of oncogenes such as Her-2/neu, c-myc, c-fos, MDM2 and p210 BCR-ABL; and (h) inactivation of p53 tumor suppressor protein by mutation or cytoplasmic sequestration of the wild-type p53. Therefore, mechanisms of resistance to chemotherapy are complex and not restricted to alterations in only a few cellular targets. Identifying novel mechanisms of resistance to chemotherapeutic agents will assist in the design of more effective strategies to overcome resistance in cancer cells. In this report, we characterize MCF-7/Adr-20, a DOX-resistant variant of the human breast cancer cell line MCF-7, with defects in the activation of the initiator and executioner caspases.

Materials and Methods

Cell lines and culture conditions. The human breast cancer cell line MCF-7 line was obtained from American Type Culture Collection (ATTC) (Manassas, VA, USA). The multidrug-resistant variant MCF-7/Adr-20 cells were isolated by stepwise selection of MCF-7 cells exposed to increasing concentrations of doxorubicin (DOX) in our laboratory and were maintained in 10 ng/ml DOX. The human leukemia cell lines HL-60 and its MRP-1-bearing variant HL-60/ADR, selected for resistance to DOX, were originally obtained from Dr. Melvin S. Center (Division of Biology, Kansas State University, Manhattan, KS, USA). The P-glycoprotein expressing HL-60/VCR cells were selected for resistance to vincristine (VCR) in our laboratory as previously described (8). HL-60/ADR and HL-60/VCR cells were maintained in 1 μg/ml DOX and VCR in growth medium, respectively. The cell lines were maintained in RPMI-1640 medium containing 10% fetal calf serum and 100 ng/ml each of penicillin and streptomycin at 37˚C in 5% CO_2. Drugs were removed from the resistant cell lines one week before each assay.

Methylene blue cell survival assay. The methylene blue cell survival assay was performed basically as previously described (9). MCF-7 or MCF-7/Adr-20 cells (1,000 cells/well in 100 μl of medium in 96-well plates) were treated with or without increasing concentrations of DOX in 5% CO_2 at 37˚C for 72 h. The cells were then fixed with 70% ethanol, stained with methylene blue and the absorbance of the dye eluted from the fixed cells in each well was measured on an automated scanning photometer at a wavelength of 630 nm. The concentration of drug that inhibited cell survival by 50% (IC_{50}) was determined from cell survival plots. Each point is the average of triplicate determinants. Fold resistance is the ratio of the IC_{50} values for the drugs used to treat MCF-7/Adr-20 cells to those of MCF-7 cells.

RNA isolation and reverse transcriptase polymerase reaction (RT-PCR) of MRP-1 and MDR1. mRNA levels of MDR1 and MRP-1 were analyzed by RT-PCR using total RNA from MCF-7, MCF-7/Adr-20, HL60/VCR and HL-60/ADR cells isolated using Tri Reagent TR-118 (Molecular Research Center, Cincinnati, OH, USA) as described by the manufacturer. One μg of total RNA was used in reverse transcription reactions with M-MLV reverse transcriptase and oligo (dT) 15 primer (Promega, Madison, WI, USA) as described by the manufacturer. The resulting total cDNA was then used as the template in PCR to measure the *MRP*-1 or *MDR*1 mRNA levels. The primers of PCR were as follows: *MRP*-1 forward 5'-TGGGACTGGAATGTCACG-3'; *MRP-1* reverse 5'-AGGAATATGCCCCGACTTC-3'; *MDR*1 forward 5'-CCCAT CATTGCAAT-3'; *MDR*1 reverse 5'-GTTCAAACTTCTGCTCC TGA-3'; β-actin forward 5'-CAGAGCAAGAGAGGCATCCT-3; β-actin reverse 5'-TTGAAGGTCTCAAACATGAT-3'. The reactions were performed at 94˚C for denaturation, 58˚C for annealing and 72˚C for extension for 30 cycles. The β-actin mRNA levels were used as internal controls. The amplified fragments were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Annexin V binding assay to detect apoptotic cells. Following treatment with 0.01, 0.1 and 1 μM DOX, the cells (5 x 10^5 cells/treatment) were used to determine the translocation of phosphatidylserine to the outer surface of the plasma membrane during apoptosis using the human phospholipid binding protein, annexin V, conjugated with fluorescein (Molecular Probes, Eugene, OR, USA) by flow cytometry as described by the manufacturer. Apoptosis and necrosis were analyzed by quadrant statistics on the propidium iodide (PI)-negative, fluorescein isothiocyanate (FITC)-positive cells and PI-positive cells, respectively.

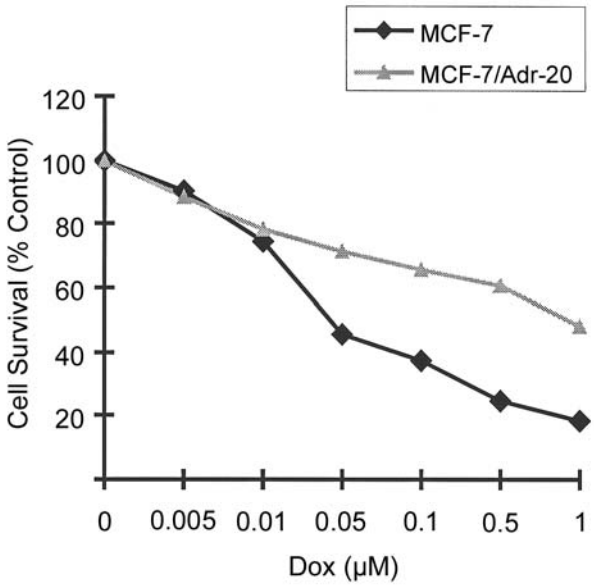

Figure 1. *Methylene blue cell survival assay. MCF-7 or MCF-7/Adr-20 cells (1,000 cells/well in 96-well plates) were treated with or without increasing concentrations of DOX at 37˚C for 72 h. The cells were then fixed with 70% ethanol, stained with methylene blue, and the absorbance of the dye eluted from the fixed cells in each well was measured on an automated scanning photometer at a wavelength of 630 nm. Each point is the average of two independent experiments and triplicate determinants in each experiment.*

Western blot analysis. Western blot analysis was performed as we described elsewhere (10) using several antibodies. In short, 50 μg protein/lane were separated by 5-15% SDS-PAGE, blotted onto a PVDF Immobilon membrane and then the protein levels were detected using the dilutions of the antibodies and peroxidase-conjugated secondary anti-rabbit, anti-mouse or anti-goat antibodies (1:2,000 v/v, Amersham, Arlington Heights, IL, USA) as described by the manufacturer. The membranes were then exposed to Kodak X-Omat film for various times. The human *MDR*1 P-glycoprotein-specific polyclonal antibody MDR-7 was produced in rabbits using a peptide sequence obtained from the deduced amino acid sequence of *MDR*1 gene. The MDR-7 antibody was used at 1:2,000 v/v. The anti-MRP-1 mouse monoclonal antibody QCRL-1 (1:1,500 w/v) was purchased from Signet Pathology System, Inc. (Dedham, MA, USA). The goat anti-caspase-7 polyclonal antibody (1:1,000 v/v) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse anti-caspase-8 polyclonal antibody (1:1,000 v/v) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-caspase-9 polyclonal antibody (1:1,000 v/v) was provided by Idun Pharmaceuticals, Inc. (La Jolla, CA, USA). The anti-caspase-6 mouse monoclonal antibody (1:1,000 v/v) and the

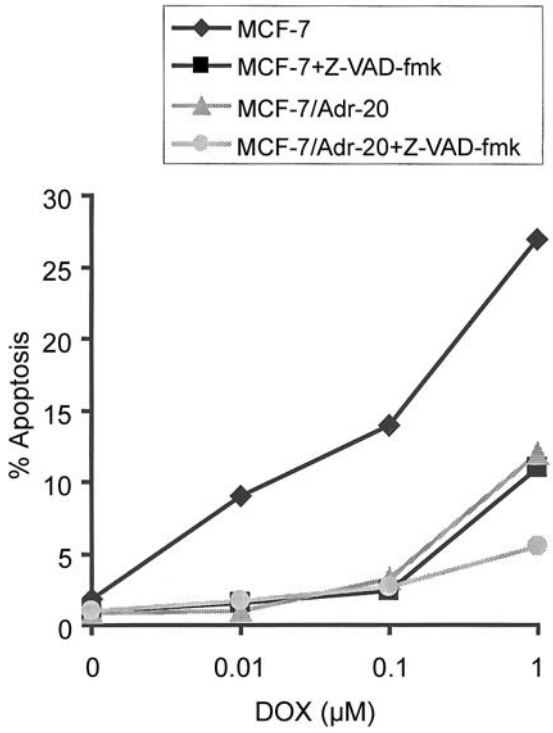

Figure 2. *Effects of DOX on the apoptosis of MCF-7 and MCF-7/Adr-20 cells. The cells (1 x 10^4/well in 96-well plates) were treated with or without increasing concentrations of DOX in the absence or presence of 100 μM Z-VAD-fmk at 37˚C for 72 h, and percent apoptosis was determined by annexin V binding assay as described in Materials and Methods. Each point is the average of triplicate determinants.*

caspase-10 monoclonal antibody (1:1,000 v/v) were purchased from Medical and Biological Laboratories Co., Ltd. (Watertown, MA, USA). p53 monoclonal antibody DO-1 (1:1,000 v/v) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The $p21^{WAF-1/CIP-1}$ monoclonal antibody (AB-1) (1:1,000 v/v) was purchased from CalBiochem (San Diego, CA, USA). The mouse monoclonal anti-β-actin antibody AC-74 (1:5,000 v/v) was obtained from Sigma (St. Louis, MO, USA).

Results

Drug resistance characteristics, P-gp and MRP1 expression in MCF-7/Adr-20 cells. MCF-7/Adr-20 was selected for resistance to DOX by stepwise selection in growth media containing increasing concentrations of DOX. The degree of resistance to DOX in the MCF-7/Adr-20 variant was analyzed by treating the cells with increasing concentrations of the drug for 72 h and performing methylene blue cytotoxicity assay. As shown in Figure 1, this drug exerted a dose-dependent cytotoxic effect on both MCF-7 and MCF-7/Adr-20 cells. To determine the degree of resistance of MCF-7/Adr-20 cells to these agents, the concentration of the drug that reduced cell survival by 50% (IC_{50}) was determined from cell survival plots. Our results show that MCF-7/Adr-20 cells are about 20-fold more resistant to DOX, compared to their parental MCF-7 cells.

To determine whether the MCF-7/Adr-20 variant expresses more resistance to apoptosis induced by DOX compared to the parental MCF-7 cells, cells were treated with 0.01, 0.1 and 1 μM DOX for 72 h and relative apoptosis was determined by annexin V binding assay. Early in the apoptotic process, phospholipid asymmetry in the plasma membrane is disrupted, leading to the exposure of phosphatidylserine on the outer leaflet of the plasma membrane. We used fluorescein-conjugated annexin V (FITC-annexin V) as described in Materials and Methods to measure apoptotic cell death in cultures of MCF-7 and MCF-7/Adr-20 cells treated with DOX. Annexin V is an anticoagulant protein that preferentially binds to negatively charged phospholipids and can be used to identify apoptosis. The data presented in Figure 2 revealed that treating MCF-7 cells with 0.01, 0.1 and 1 μM DOX for 72 h induced significantly more apoptosis than the MCF-7/Adr-20 cells treated with the same concentrations of DOX. Quantitative analysis of these data clearly show that DOX at 0.01, 0.1 and 1 μM induced 8, 14 and 27% apoptosis in MCF-7 cells. However, treatment of MCF-7/Adr-20 cells with 0.01 and 0.1 DOX for 72 h did not result in significant apoptosis and 1 μM DOX was able to trigger apoptosis in only 8% of MCF-7/Adr-20 cells (Figure 2).

To investigate whether caspases participate in DOX-induced apoptosis in MCF-7 and MCF-7/Adr-20 cells, we treated the cells with and without 100 μM Z-VAD-fmk, a very efficient inhibitor of all caspases (11, 12), for 3 h before treating the cells with 0.01, 0.1 and 1 μM DOX for 72 h. The results presented in Figure 2 show that Z-VAD-fmk almost totally inhibited DOX-induced apoptosis in MCF-7 cells treated with 0.01 and 0.1 μM DOX for 72 h. However, at 1 μM DOX treatment, Z-VAD-fmk inhibited only about 65% of apoptosis (Figure 2), indicating that apoptosis at this concentration of DOX in MCF-7 cells is only partially dependent on caspases and that caspase-independent apoptosis is also evident. Furthermore, treatment of MCF-7/Adr-20 cells with 100 μM Z-VAD-fmk and 1 μM DOX decreased apoptosis from 8% to 5% (Figure 2).

To determine whether multidrug resistance in MCF-7/Adr-20 cells is the result of P-gp and/or MRP1 overexpression, we examined the levels of expression of the *MDR*1 and *MRP*1 genes in MCF-7 and MCF-7/Adr-20 cells by Western blotting (Figure 3A and B) and RT-PCR (Figure 3C). Western blotting using the anti-P-gp antibody MDR-7 and RT-PCR analysis of the *MDR*1 gene, showed that neither P-gp nor its mRNA were detectable in these cells (Figure 3A and C). As a positive control in these experiments, P-gp-bearing HL-60/VCR cells, an MDR

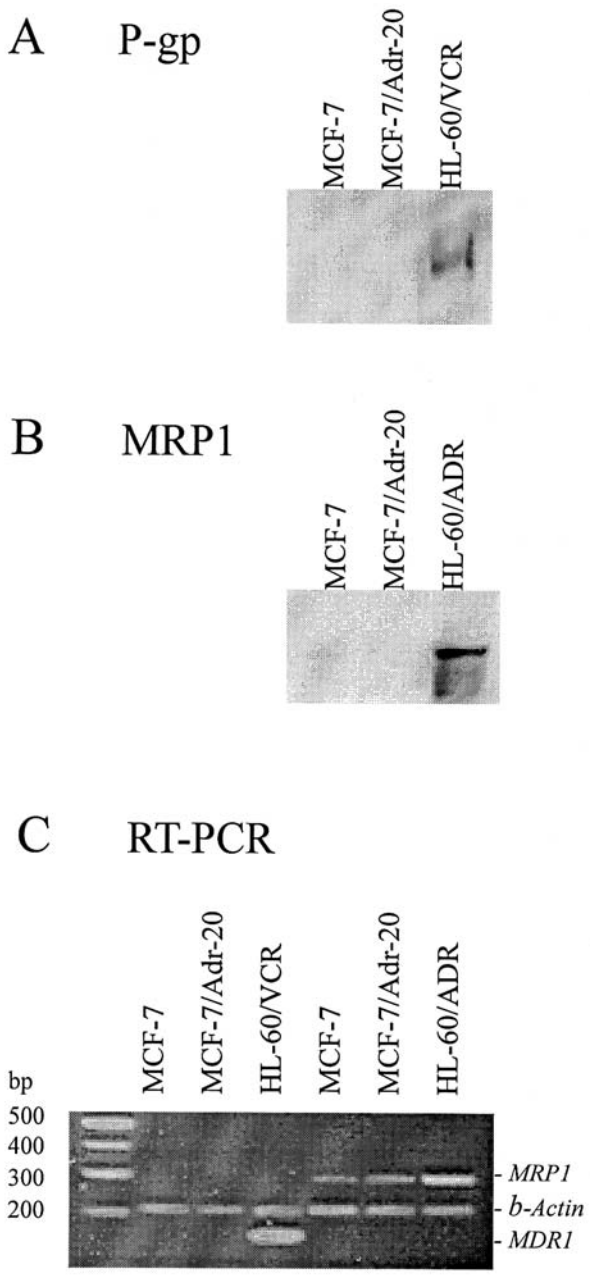

Figure 3. *Analysis of expression of P-gp and MRP1 genes in MCF-7 and MCF-7/Adr-20 cells. For Western blot analysis, cells (1 x 10^5/lane) were subjected to 5-15% SDS-PAGE, and P-gp (A), MRP1 (B) protein levels were detected by using antibodies to these proteins and the ECL protein detection kit (Amersham Pharmacia Biotech, Inc. Piscataway, NJ, USA) as described in Materials and Methods. As positive controls, the P-gp overexpressing MDR cell line, HL-60/VCR (lane 3), and the MRP1 overexpressing drug resistant variant HL-60/ADR (lane 6) were used. (C) RT-PCR analysis of expression of MDR1 (lanes 1 and 2) and MRP1(lanes 4 and 5) genes in MCF-7 and MCF-7/Adr-20 cells compared to the β-actin mRNA levels in these cells as internal controls. As positive controls, HL-60/VCR (lane 3) and the HL-60/ADR (lane 6) were used. The RT-PCR products were run on 1.5% agarose gels and visualized by EtBr staining. Lane 1 contains Hae III-cut φX174 DNA fragments used as molecular weight markers.*

variant of HL-60 cells, were used. RT-PCR of the *MRP*1 gene showed similar expression levels of *MRP*1 mRNA in both MCF-7 and MCF-7/Adr-20 cells (Figure 3C). Moreover, using the anti-MRP 1 antibody QCRL-1, MRP1 was not detected in these cells (Figure 3B). As a positive control, MRP1-overexpressing HL-60/ADR cells were used in these experiments (Figure 3B and C).

Analysis of DOX-induced caspase activation in MCF-7 and MCF-7/Adr-20 cells. It is well-documented that apoptosis induced by different stimuli, such as death ligands, chemotherapeutic drugs or ionizing irradiation, leads to the activation of caspases by proteolytic cleavage (13). Furthermore, some of these death stimuli can also cause caspase-independent apoptosis (14). Therefore, we examined (a) whether DOX-induced apoptosis in MCF-7 cells is associated with the activation of caspases-6, -7, -8 and -10 and (b) whether the decreased level of apoptosis triggered by DOX in MCF-7/Adr-20 cells is because of the lack of activation of these caspases. These experiments were performed by treating MCF-7 and MCF-7/Adr-20 cells with 0.01, 0.1 and 1 μM DOX for 72 h, respectively and evaluating the activation of caspases-6, -7, -8 and -10 by Western blot analysis using antibodies known to recognize the proforms, as well as the active forms, of these caspases. Western blot analysis of the initiator caspase-8 revealed that 0.01 and 0.1 μM DOX treatment for 72 h did not affect procaspase-8, but 1 μM DOX treatment induced cleavage and activation of the procaspase-8 to its 43 kDa active form in MCF-7 (Figure 4A). However, the same concentrations of DOX did not affect procaspase-8 in MCF-7/Adr-5 cells. Additionally, DOX at 1 μM induced degradation of procaspase-9 to its active 37 kDa active form in MCF-7 cells, but had no effect on procaspase-9 in MCF-7/Adr-20 cells (Figure 4B). Furthermore, Western blot analysis also revealed that the initiator caspase-10 was processed to its active form only at 1 μM DOX treatment in MCF-7 cells, but was not activated in MCF-7/Adr-20 cells (Figure 4C).

Western blot analysis of the executioner caspase-6 showed that the anti-caspase-6 antibody detected the major 34 kDa procaspase-6 in both MCF-7 and MCF-7/Adr-20 cells. Moreover, treating the cells with 0.01 and 0.1 μM DOX for 72 h did not cause processing and activation of the 34 kDa procaspase-6, but 1 μM DOX treatment resulted in generation of the active 20 kDa form of caspase-6 in MCF-7 cells (Figure 5A). Moreover, Western blot analysis of the executioner caspase-7 revealed that 0.1 and 1 μM DOX treatment cleaved procaspase-7 to its activated 20 kDa form in MCF-7 cells, but did not cleave or activate procaspase-7 to its active form in MCF-7/Adr-20 cells (Figure 5B).

To determine whether DOX treatment induces p53 and $p21^{WAF-1/CIP-1}$ during apoptosis, MCF-7 and MCF-7/Adr-20 cells were treated with 0.01, 0.1 and 1 μM DOX for 72 h

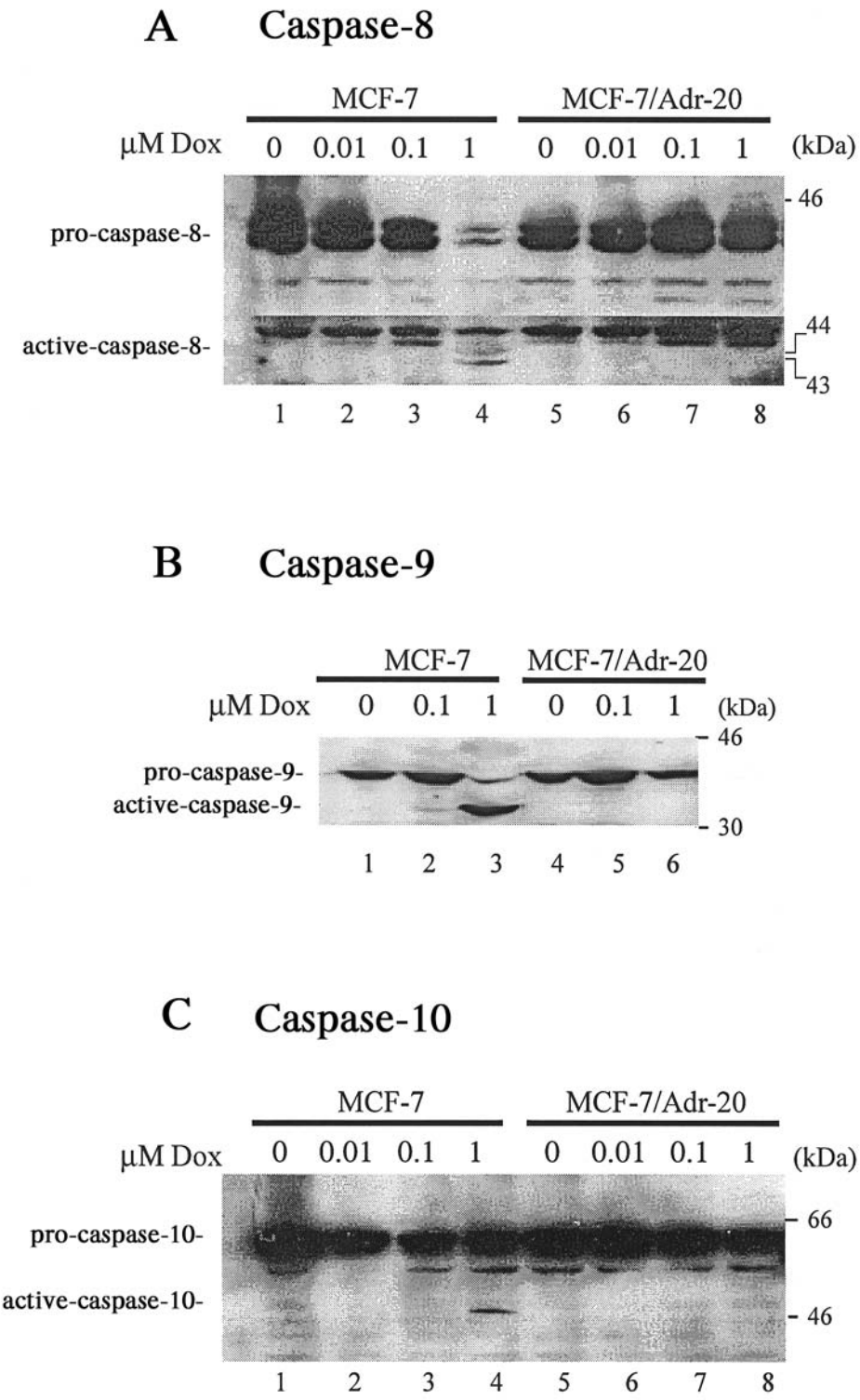

Figure 4. *Effects of DOX treatment on initiator caspases-8, -9 and -10. (A-C) Aliquots (50 μg protein) from untreated MCF-7 and MCF-7/Adr-20 cells (lanes 1 and 5), MCF-7 cells (lanes 2-4), and MCF-7/Adr-20 cells (lanes 6-8) treated with 0.01, 0.1 and 1 μM DOX, respectively for 72 h at 37°C were processed for Western blot analysis using antibodies to caspases-8, -9 and -10, respectively, as described in Materials and Methods.*

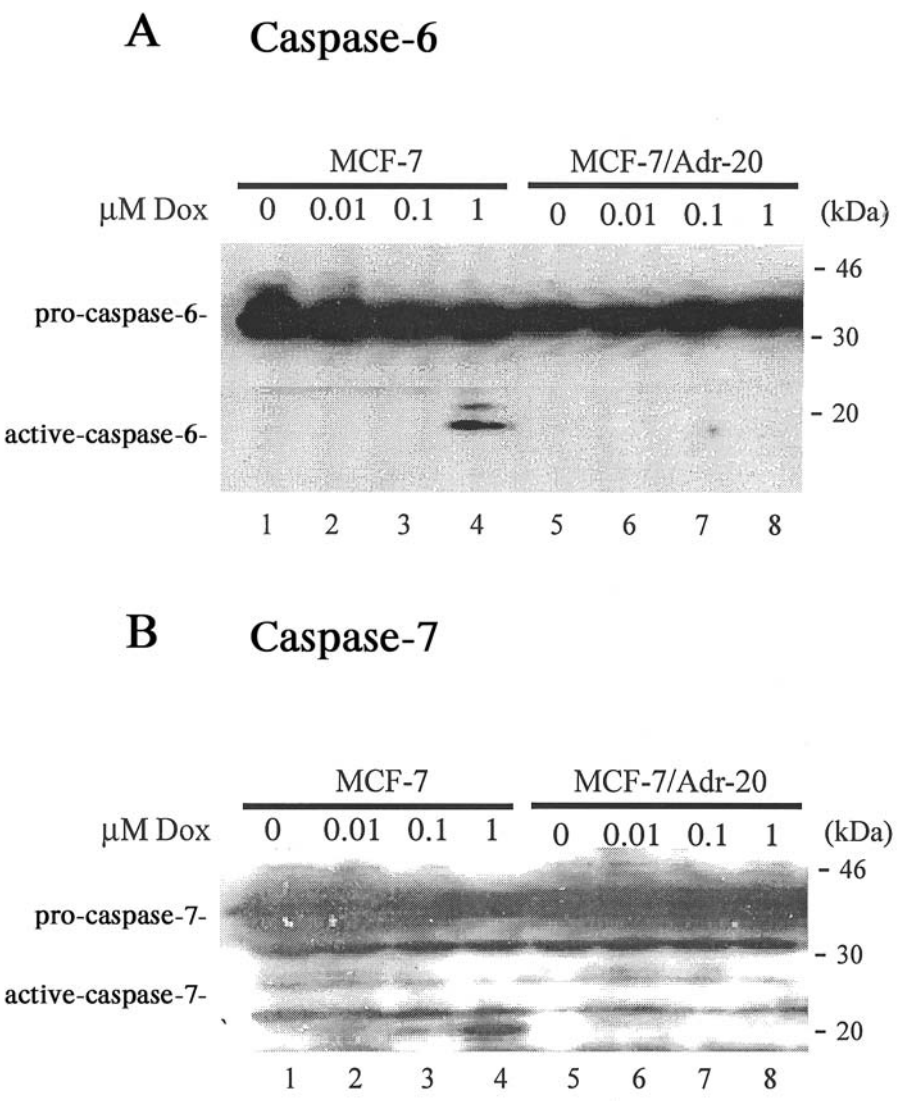

Figure 5. *Effects of DOX treatment on initiator caspases-6 and -7. (A and B) Aliquots (50 μg protein) from untreated MCF-7 and MCF-7/Adr-20 cells (lanes 1 and 5, respectively), MCF-7 cells (lanes 2-4), and MCF-7/Adr-20 cells (lanes 6-8) treated with 0.01, 0.1 and 1 μM DOX, respectively, for 72 h at 37˚C were processed for Western blot analysis using antibodies to caspase-6 (A) and caspase-7 (B) as described in Materials and Methods.*

and levels of these proteins were detected by Western blotting. The data in Figure 6 clearly show that p53 was induced to the same extent by DOX at the concentrations used in both the MCF-7 and MCF-7/Adr-20 cells. Interestingly, while 0.01 and 0.1 μM DOX induced $p21^{WAF-1/CIP-1}$ in both MCF-7 and MCF-7/Adr-20 cells, 1 μM DOX induced this protein in MCF-7/Adr-20 cells, but not in MCF-7 cells.

Discussion

In the present study, we characterized MCF-7/Adr-20, a DOX-resistant variant of the human breast cancer cell line MCF-7. This drug-resistant variant expresses about 20-fold resistance to DOX, but does not overexpress either P-gp or MRP1. Our data revealed that 72-h treatment with 0.01 and 0.1 μM DOX induced primarily caspase-dependent apoptosis in MCF-7 cells and at 1 mM, DOX caused both caspase-dependent and -independent apoptosis in these cells. In general, two major pathways are involved in apoptosis: (a) the mitochondrion-initiated pathway and (b) the cell surface death receptors pathway (15-17). In the mitochondrial pathway, cytochrome c, certain caspases, apoptosis-inducing factor (AIF), Smac/DIABLO, inhibitor of apoptosis protein (IAP)-binding protein and other apoptosis-inducing factors are released from the

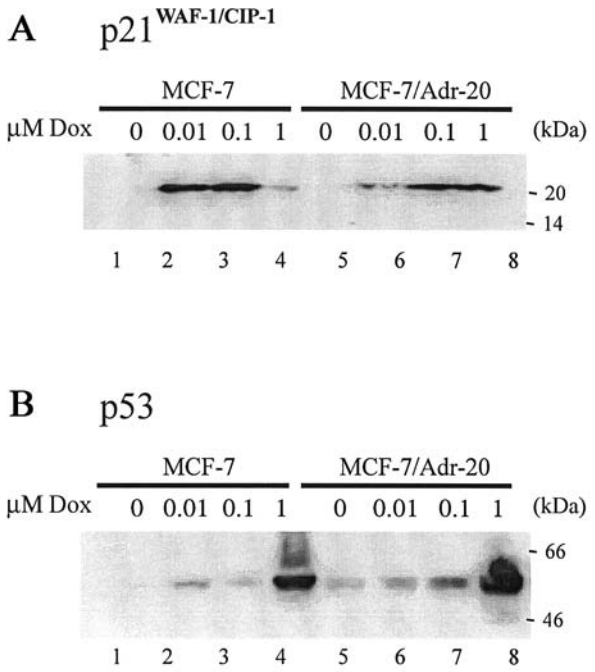

Figure 6. Western blot analysis of p53 and p21$^{WAF-1/CIP-1}$ in cells treated with Taxol. Aliquots (50 μg protein) from untreated MCF-7 and MCF-7/Adr-20 cells (lanes 1 and 2, respectively), MCF-7 cells (lanes 2-4), and MCF-7/Adr-20 cells (lanes 6-8) treated with 0.01, 0.1 and 1 μM DOX, respectively, for 72 h at 37˚C, were processed for Western blot analysis using antibodies to p53 (A), and p21$^{WAF-1/CIP-1}$(B) as described in Materials and Methods.

intramembrane space to the cytosol (18). Once released, cytochrome c, together with dATP, binds to apoptotic proteinase activating factor-1 (Apaf-1) and this complex, along with adenine nucleotides, promotes procaspase-9 autoactivation (19), which in turn activates caspases-2, -3, -6, -8 and -10 (20-22). In the death receptor-mediated apoptosis pathway (Fas/Fas ligand interaction and cell death), caspases-8 and -10 are initiator caspases that can activate the downstream caspases including caspases-3, -6 and -7. Active caspase-8 also cleaves the pro-apoptotic protein Bid and the truncated Bid induces mitochondrial cytochrome c release (19, 23), thereby linking the two pathways. After activation, both caspase-8 and caspase-9 activate caspase-3, which in turn cleaves other caspases and many cellular proteins (13, 14, 22).

Caspase-dependent apoptosis in the MCF-7 cell line is independent of caspase-3, since it is known that MCF-7 cells lack caspase-3 expression as a result of a functional deletion mutation in the caspase-3 gene (23). Consistent with our results, other investigators have also shown that DOX-triggered apoptosis in MCF-7 cells was associated with the release of cytochrome c from the mitochondria (25-27) and PARP degradation, which was blocked by Z-VAD-fmk, the general caspase inhibitor (25, 26). However, in MCF-7/Adr-20 cells, DOX induced apoptosis only at 1 mM treatment for 72 h. Since DOX-induced apoptosis in MCF-7 cells was primarily caspase-dependent, we examined (a) whether DOX-induced apoptosis in MCF-7 cells is associated with the activation of caspases-6, -7, -8 and -10 and (b) whether the decreased level of apoptosis triggered by DOX in MCF-7/Adr-20 cells is due to the lack of activation of these caspases.

Molecular mechanisms of defects in caspase activation by DOX in MCF-7/Adr-20 cells remain to be found. However, several factors, including low expression or absence of Apaf-1 (28), deficiency in intracellular calcium pools (29), increased expression of IAPs (7, 30), or up-regulation of caspase-8 inhibitors like FLIP (FLICE-like Inhibitory Protein) (31) may contribute to the lack of caspase activation in this drug-resistant variant. It is well established that IAPs, the endogenous caspase inhibitors, must be degraded by the proteasome for progression of apoptosis (32, 33). The IAP family of proteins contains baculoviral IAP repeat (BIR)-domains (34), which bind to and inhibit caspases and as a result rescue cells from apoptosis. Several members of this family (*e.g.*, XIAP, c-IAP-1 and Survivin) are frequently up-regulated in tumor cells and they cause resistance to cancer therapy due to inhibition of tumor cell apoptosis (7, 29, 35). IAPs may be more important in mitochondrion-induced cell death, since they interact with caspases-3, -6, -7 and -9, but not with caspase-8 (36).

Our results showed that treatment with 0.01 and 0.1 μM DOX for 72 h significantly increased p21$^{WAF-1/CIP-1}$ in MCF-7 and MCF-7/Adr-20 cells compared to control untreated cells. However, treating MCF-7 cells with 1 μM DOX decreased the level of p21$^{WAF-1/CIP-1}$ expression to the level measured in untreated control cells. Interestingly, 1 mM DOX treatment significantly increased the level of p21$^{WAF-1/CIP-1}$ in MCF-7/Adr-20 cells. Whether DOX-induced activation of caspases results in degradation and down-regulation of p21$^{WAF-1/CIP-1}$ in MCF-7 cells remains to be found. In light of this, a recent report showed that at low concentration, DOX-induced p21$^{WAF-1/CIP-1}$ in the SKN-SH neuroblastoma cell line was associated with senescence and, at a higher concentration, it induced activation of caspase-3, which caused degradation of p21$^{WAF-1/CIP-1}$ (37). Moreover, p53 expression in both cell lines treated with various concentrations of DOX increased significantly compared to untreated controls. Evidence indicates that subapoptotic concentrations of cytotoxic drugs can induce growth arrest with senescence features and that p53 and p21$^{WAF-1/CIP-1}$ are major players in this process (38, 39). Furthermore, in addition to its role as a cell cycle regulator, p21$^{WAF-1/CIP-1}$ functions to prevent apoptosis, mediating the survival function of phosphatidylinositol (PI3K) through phosphorylation of AKT (40, 41). Recently, Fan *et al.* (42) reported that an antisense

oligonucleotide to $p21^{WAF-1/CIP-1}$ caused apoptosis of MCF-7 cells, revealing the survival function of this protein in this cell line. It is possible that $p21^{WAF-1/CIP-1}$ functions in protecting DNA-damaged cells from becoming apoptotic, while p53-mediated DNA repair is in process.

In conclusion, we have selected a DOX-resistant variant of the MCF-7 cell line, MCF-7/Adr-20, which does not overexpress P-gp or MRP1 and is defective in DOX-induced activation of caspases-6, -7, -8, -9 and -10 compared to MCF-7 cells. Moreover, our results suggest that the lack of activation of these caspases in MCF-7/Adr-20 cells may prevent degradation of $p21^{WAF-1/CIP-1}$, which in turn may be involved in resistance to apoptosis. Further elucidation of the molecular mechanisms regulating the lack of activation of these caspases in MCF-7/Adr-20 cells could potentially provide important insights and will help in developing strategies to modulate drug resistance.

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