Forced Expression of Heat Shock Protein 27 (Hsp27) Reverses P-Glycoprotein (ABCB1)-mediated Drug Efflux and MDR1 Gene Expression in Adriamycin-resistant Human Breast Cancer Cells

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Mutant p53 accumulation has been shown to induce the multidrug resistance gene (MDR1) and ATP binding cassette (ABC)-based drug efflux in human breast cancer cells. In the present work, we have found that transcriptional activation of the oxidative stress-responsive heat shock factor 1 (HSF-1) and expression of heat shock proteins, including Hsp27, which is normally known to augment proteasomal p53 degradation, are inhibited in Adriamycin (doxorubicin)-resistant MCF-7 cells (MCF-7/adr). Such an endogenous inhibition of HSF-1 and Hsp27 in turn results in p53 mutation with gain of function in its transcriptional activity and accumulation in MCF-7/adr. Also, lack of HSF-1 enhances nuclear factor κB (NF-κB) DNA binding activity together with mutant p53 and induces MDR1 gene and P-glycoprotein (P-gp, ABCB1), resulting in a multidrug-resistant phenotype. Ectopic expression of Hsp27, however, significantly depleted both mutant p53 and NF-κB (p65), reversed the drug resistance by inhibiting MDR1/P-gp expression in MCF-7/adr cells, and induced cell death by increased G2/M population and apoptosis. We conclude from these results that HSF-1 inhibition and depletion of Hsp27 is a trigger, at least in part, for the accumulation of transcriptionally active mutant p53, which can either directly or NF-κB-dependently induce an MDR1/P-gp phenotype in MCF-7 cells. Upon Hsp27 overexpression, this pathway is abrogated, and the acquired multidrug resistance is significantly abolished so that MCF-7/adr cells are sensitized to Dox. Thus, clinical alteration in Hsp27 or NF-κB level will be a potential approach to circumvent drug resistance in breast cancer.

Development of a multidrug-resistant phenotype is a major obstacle to the successful treatment of breast cancer (1, 2). There are two major pathways by which cancer cells acquire drug resistance, drug efflux and direct suppression of apoptosis. Drug efflux is due to increased plasma membrane accumulation of various ATP-binding cassette (ABC)2 transporters, including ABCB1, also known as P-glycoprotein (P-gp), which extrude the internalized drugs from the cancer cells (3–5). Various approaches have been reported to overcome the drug efflux, including pharmacological inhibition of ABCs and modulation of endogenous regulators of MDR1 (6). Drug resistance is also acquired via direct suppression of apoptotic pathways due to accumulation of mutant p53 (mutp53) with “gain of function” (7, 8) and increased expression of antiapoptotic proteins, such as BCI-2 (4). In addition to abrogating the proapoptotic function of wild type p53 (wtp53), these p53 missense mutations have been shown to have unusual gain of function properties both in vitro and in vivo (9–11). Recent studies have established a link between these two pathways of drug resistance (12).

Mutant p53 has been found to be the prominent common mediator of both pathways (11). Wild type p53 is generally known to repress the expression of the MDR1 gene, which codes for the ABC protein P-gp through interaction with basal transcription factors, such as TATA-binding protein (13). Interestingly, mutant p53 (both in the C and N termini) is still capable of interacting with TATA-binding protein but is unable to repress MDR1 transcription (14, 15). Indeed, mutant p53 has been demonstrated to activate the MDR1 promoter probably by both dominant negative and gain of function mechanisms (16). However, understanding of the roles of other p53-dependent mediators of drug resistance, especially mediators of ABC induction, in breast cancer cells still remains elusive. In particular, the role of other co-effectors of MDR1 gene induction by mutant p53 needs to be identified for complete understanding of the MDR1 gene expression in MCF-7/adr cells.

One of the potential mechanisms by which mutant p53 is involved in the regulation of MDR1 is via the activation of nuclear factor κB (NF-κB) (17). Previous studies have illustrated that NF-κB can independently activate MDR1 (18–20). A binding site (CAAT) for NF-κB has been identified in the MDR1 promoter and has been proven to be involved in the transcriptional activation of MDR1 (21, 22). Moreover, mutp53

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2 The abbreviations used are: ABC, ATP binding cassette; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Dox, doxorubicin; CMAC, 7-amino-4-chloromethylcoumarin; EMSSA, electrolytic mobility supershift assay(s); SSBD, sequence-specific DNA binding domain; CTD, C-terminal domain.
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expression was found to correlate positively with NF-κB activity in cancer cells (23, 24), even without external triggers. Constitutive NF-κB activity can be augmented by mutant p53 to transactivate the NF-κB2 gene, encoding the p100/p52 subunit of NF-κB (24, 25). Additionally, mutp53 was observed to have a pronounced effect on nuclear accumulation and retention of RelA (p65) upon cytokine exposure, and a strong correlation between mutp53 overexpression and nuclear p65 staining was demonstrated in tumors (26). Thus, a portion of the gain of function for mutp53 may involve a mutp53-dependent enhanced NF-κB oncogenic activity leading to the activation of the MDR1 gene. Hence, mutant p53 and NF-κB may have a strong relationship with other stress-responsive factors in the overall multidrug resistance scheme.

Heat shock factor 1 (HSF-1) is a multifaceted factor that can participate in many stress-related transcriptional and translational activities in eukaryotic cells by binding to heat shock elements (HSE) in the promoters of many genes (27). Although HSF-1 is primarily associated with expression of heat shock proteins, it has also been found to be activated in response to oxidative stress, DNA damage, and other stress stimuli and to play key roles in stress-induced cell signaling cascades. Transcriptional activation of HSF-1 is complex, and it is regulated by multiple steps, including phosphorylations, trimerization, and translocation to the nucleus as well as acetylation/deacetylations by p300 and SIRT1 in the chromatin-bound state (28). Because Dox is a DNA-damaging agent, it would be expected to activate HSF-1, but not much is known in regard to the role of HSF-1 in response to Dox. The MDR1 promoter region has been shown to have HSE, and HSF-1 can repress MDR1 gene expression (29); however, it is not known how drug-induced HSF-1 activation regulates MDR1 gene expression in cancer cells. Although heat-shock proteins have ubiquitous functions (30), they could play a general role in the development of drug resistance. Expression of the small heat shock protein 27 (Hsp27) is associated with the response of cancer cells to heat shock and chemotherapeutic drugs, through HSF-1 activation (31, 32). Transient Hsp27 overexpression has been found to induce drug resistance by activating antiapoptotic pathways in different cancer models (33–36). However, the role of Hsp27 in the regulation of the ABC transporters is not clearly understood.

We hypothesized that repeated and chronic exposure of cancer cells to Dox will silence the HSF-1 gene, and this response may suppress expression of Hsp proteins with time, including Hsp27, which is critical in p53 protein homeostasis, and induce the MDR1 gene in MCF-7/adr cells. As such, overexpression of Hsp27 could potentially reverse the drug resistance in these cells. We have used MCF-7/adr cells as a model of drug resistance phenotype to test our hypothesis. Our results have shown that HSF-1 is very poorly expressed in MCF-7/adr cells, compared with MCF-7 cells, resulting in decreased expression of Hsp27. This was accompanied by an increase in p53, NF-κB, and P-gp. Hsp27 overexpression reversed the P-gp-mediated drug efflux and increased the Dox sensitivity in MCF-7/adr cells. We provide evidence for a novel mechanism involving Hsp27, mutp53, and NF-κB in the regulation of MDR1 gene expression.

MATERIALS AND METHODS

Cell Culture and Treatments—MCF-7/adr cells, derived from the parental drug-sensitive MCF-7 cells by stepwise selection with Dox, were kindly provided by Dr. Kapil Mehta (University of Texas M.D. Anderson Cancer Center, Houston, TX). To maintain the drug resistance phenotype, this cell was cultured in the presence of 1 μg/ml Dox in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 10 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were maintained at 37 °C in 5% CO₂, 95% air.

Toxicity Measurements (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay)—Cell viability against Dox-induced toxicity was determined using an MTT assay in 96-well plates. MCF-7/adr cells were transfected with Hsp27-overexpressing vector or empty vector. 2000 cells in 300 μl of medium were added to each well (quadruplicate) in 96-well flat bottom tissue culture plates and treated with the desired concentration of Dox (in the range of 0.5, 1, 5, and 10 μg/ml). After a desired post-drug treatment time (24 or 48 h), an MTT assay was carried out as described previously (37).

Plasmids and Transfections—The plasmid pCMV-SPORT6/Hsp27 was obtained by subcloning the full-length human Hsp27 cDNA (36) into the mammalian expression vector pCMV-SPORT6 (Open Biosystems). The gene was under control of the CMV promoter, allowing for constitutive expression of Hsp27. MCF-7/adr cells (2 × 10⁶ cells/plate) were seeded in 100-mm plates and cultured for 24 h before transfection. Cells were transfected with pCMV-SPORT6/Hsp27 or control plasmids by mixing DharmaFect 4 transfection agent (20 μl) (Ambion Inc.) and plasmid (20 μg) in antibiotics free and reduced serum medium for 20 min at room temperature and then adding the mix to the cells. After overnight incubation, the cells were washed twice in medium and then incubated in fresh medium for 24 h before treating with drug for the desired time before harvesting for analysis.

Preparation of Whole Cell Lysates and Western Blot Analysis—For whole cell lysates, the cells were washed twice with ice-cold PBS, lysed in radioimmunoprecipitation assay lysis buffer (0.368 mg/ml sodium orthovanadate, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 17 μg/ml aprotonin), and incubated on ice for 45 min, followed by centrifugation at 12,000 rpm to remove cellular debris. Cytosolic and nuclear proteins were isolated using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) as follows. Cells were finely minced in cold cytosolic buffer and centrifuged twice at 1500 × g, and then the combined pellet was collected for nuclear isolation. Supernatant was centrifuged again at 20,000 × g for 20 min and was collected as the cytosolic fraction. Nuclear pellets were resuspended and homogenized with 10 volumes of nuclear buffer A (10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 0.1 mM EDTA, 3.3 mM CaCl₂, 0.1 mM PMSE, 0.5 mM DTT, and 2.4 mM sucrose) after centrifugation at 20,000 × g for 10 min at 4 °C, and supernatant was collected for use. 60 μg of total protein was resolved by 4–12% SDS-PAGE, unless stated otherwise, and transferred to PVDF membrane. Western blot analysis was performed using primary antibodies for Hsp27, phospho-Hsp27...
iodide 10 stain (10 body prepared in blocking buffer on the coverslips for 1 h at addition of primary mouse monoclonal NF-κB p65, NF-κB p50, IκBα, IKKα (Cell Signaling, Beverly, MA), and P-gp (Abcam Inc., Cambridge, MA) and detected using the appropriate HRP-labeled secondary antibody and enhanced chemiluminescence (Pierce). Each Western blot shown is representative of at least three separate experiments.

**Dox Accumulation Analysis by Confocal Microscopic Fluorescence Imaging**—To examine the intracellular distribution of Dox in MCF-7/adr cells, we used scanning confocal microscopy (Zeiss LSM 510). The cells were cultured on coverslips treated with Dox for the desired time, washed with PBS, and examined using a confocal microscope. Images were collected using an LSM510 NLO confocal microscope with a C-Apo ×20 water immersion lens (Carl Zeiss, Jena, Germany). Dox fluorescence was determined by exciting with an argon laser at 488 nm, and the emission was collected through a 530-nm long pass filter. The same confocal settings (excitation, laser power, detector, gain, and pin hole) were used to image the MCF-7/adr- and Hsp27-overexpressing MCF-7/adr cells. Post-data acquisition image analysis was performed using MetaMorph software (Molecular Devices, Downingtown, PA). Cell images were analyzed as mean doxorubicin fluorescent intensity per pixel in a circular 314-pixel region of the nucleus or cytoplasm of the same cell (−60 μm²). Multiple fields containing at least 15 cells/field were imaged in each experiment.

**Analysis of NF-κB p65 Translocation Using Immunofluorescence Microimaging**—MCF-7/adr cells were grown for 24 h (with and without Hsp27 overexpression) on coverslips and treated with Dox at different concentrations for 48 h, and then the cells were washed with PBS, fixed, and permeabilized for 30 min with 4% paraformaldehyde in PBS with 0.25% Triton X-100. Cells were blocked with 5% BSA in PBS containing 0.01% Tween 20 for 1 h at room temperature, followed by the addition of primary mouse monoclonal NF-κB p65 (1:50) antibody prepared in blocking buffer on the coverslips for 1 h at room temperature. After washing four times with PBS containing Tween 20 at 0.05%, Alexafluor 488 tag conjugated goat anti-mouse IgG (1:300) secondary antibody was added with Hoechst stain (10 μl/ml) for nuclei, and the slides were incubated at room temperature for 1 h. Images were acquired at room temperature with an LSM510 NLO confocal microscope with a C-Apo ×20 and ×60 water immersion lens (Carl Zeiss, Jena, Germany). Post-data acquisition image analysis was performed using MetaMorph software (Molecular Devices). Cell images were analyzed as mean Alexafluor 488 intensity per pixel in a circular 314-pixel region of the nucleus or cytoplasm of the same cell (−60 μm²). Multiple fields containing at least 15 cells/field were imaged in each experiment.

**Cell Cycle Analysis**—Control and Dox-treated MCF-7/adr cells with or without Hsp27 overexpression were harvested at the indicated times, washed in ice-cold PBS, fixed in 2.0 ml of ice-cold ethanol (75%), and left at −20 °C overnight. After overnight fixation, cells were washed and resuspended in 1.0 ml of PBS. Further, DNase-free bovine pancreas RNase (Sigma-Aldrich; 10 μl, final concentration 1 mg/ml) and propidium iodide 10 μl (10 mg/ml) were added to 1.0 ml of cell suspension in the dark. Cell suspensions were mixed and incubated at 37 °C for 30 min and analyzed using a BD FACSCalibur at 617-nm emission wavelength. The DNA content-frequency histogram was analyzed using FlowJo version 8.8.6 software and used to calculate the percentage of cells in respective phases of the cell cycle.

**Intracellular Dox and Cell Tracker Blue (7-Amino-4-chloromethylcoumarin; CMAC) Measurements Using FACS Analysis**—One million cells of MCF-7/adr or MCF-7/adr/Hsp27 were treated with Dox alone (1, 5, and 10 μg/ml) or Dox + 25 μM CMAC for 48 h. The intracellular fluorescence of Dox and CMAC were measured using a FACScan flow cytometer (BD Biosciences). The parameters of fluorescence recording for Dox were as follows: excitation wavelength, 488 nm; emission wavelength, 576 nm; shutter 100; rate, 500 cells/s; number of analyzed events, 10,000. For CMAC, excitation and emission wavelengths were 353 and 466 nm, respectively. Histograms were analyzed using the FlowJo version 8.8.6 software by the parameters of average fluorescence of cells in the population studied.

**Reverse Transcription PCR for P-gp and HSF-1 mRNA Determination**—The mRNA levels of HSF-1 and P-gp in control MCF-7 and MCF-7/adr cells were determined by RT-PCR. Total RNA was extracted using the standard procedure provided by the manufacturer.

**Electrolytic Mobility Shift and Supershift Assay (EMSA and EMSSA)**—The cells were collected from control and different post-drug treatment time points, and nuclear proteins were extracted using a nuclear protein extraction kit (NE-PER) from Pierce. EMSA for HSF-1 was performed using an oligonucleotide probe corresponding to a double-stranded HSE consensus sequence, labeled with biotin on the 5'-end (5'-biotin-CTAGAAGCTTCTAGAAGCTTCTAG-3'). For each sample, 15 μg of extracted nuclear protein was added with 20 fmol of biotin-HSE, 2.5% glycerol, 50 ng/μl poly(dI-dC), and 5 mM MgCl₂, provided in the EMSA kit (Pierce). In control experiments, an HSE oligonucleotide devoid of biotin tag was used. In EMSSA assays, monoclonal HSF-1 antibody was used. This sample complex was incubated at room temperature for 20 min, and 5× loading buffer was added. The prepared samples were resolved by electrophoresis on 6% precast DNA retardation gel (Invitrogen) and transferred to a nylon membrane. The transferred DNA was cross-linked to the membrane with a handheld UV lamp equipped with 254-nm bulbs for 5 min at a distance −0.5 cm from the membrane. Finally, the biotin-labeled DNA was detected by chemiluminescence as per the protocol in the chemiluminescent nucleic acid detection module (Pierce).

**Luciferase Activity Assays**—Two different MDRI promoter fragments were generated from MCF-7/adr cell genomic DNA as a template (21, 22). Wild type MDRI promoter fragment spanning 198 to +43 and mutant MDRI promoter fragment −155 to +43 (HSF-1 binding motif deletion) were generated using the primers (supplemental Tables 1 and 2) with HindIII restriction sites. The PCR amplification products were digested with HindIII, inserted into pGL3B (Promega, Madison, WI), and sequenced (supplemental Fig. 3). Transfection was performed using the LipofectamineTM reagent (Invitrogen). Cells were seeded in 10-cm plates at a density of 10⁵ cells/plate. The next day, the cells were incubated for 5 h with 1 ml of reduced...
serum medium containing 5 μg of pGL3B, wild type MDR1-luc, and mutant MDR1-luc plasmids. Following transfection, the mixture was removed, and the cells were incubated under standard conditions for 24 h, after which cells were harvested and subjected to luciferase activity assays by using a Luciferase Assay System Freezer Pack kit (Promega) and a Centro XS3 Luminometer (Berthold Technologies). The values were normalized to protein concentration. Normalization of the transfection efficacy was performed using a chemiluminescent reporter gene assay system to detect β-galactosidase (Tropix Inc., Bedford, MA).

Data Analysis—Data analysis was performed using Student’s t test and one-way analysis of variance. Data are presented as means ± S.E. The general acceptance level of significance was p < 0.05.

RESULTS

Inhibition of HSF-1, Depletion of Hsp27, and Accumulation of Mutant p53 and NF-κB in MCF-7 and MCF-7/adr Cells—In Dox-sensitive MCF-7 cells, the basal level of HSF-1 and its target protein, Hsp27, were found to be significant, whereas there were only trace amounts of HSF-1 and Hsp27 detected in the Dox-resis-tant MCF-7/adr cells (Fig. 1A). This is contrary to the largely known fact that Hsp27 overexpression can cause drug resistance in cancer cells (38). Hsp70, another antiapoptotic Hsp, did not show any significant difference between MCF-7 and MCF-7/adr cells. Because Hsp27 is known to accelerate proteasomal degradation of wtp53 in cancer cells (39), we determined the levels of p53 expression. A large increase in p53 was observed in MCF-7/adr cells at resting conditions, compared with the Dox-sensitive MCF-7 cells, which had no detectable p53 under identical conditions (Fig. 1A). Because p53 is a ubiquitous protein, at normal conditions there will not be any wtp53 detectable (40). The detected p53 in MCF-7/adr cells has been characterized previously (21, 22) as mutant p53 with the P72R point mutation and deletion of 126–133 residues but with retention of the full C-terminal domain (nuclear localization signal) (22). The absence of Hsp27 probably contributes to the stability through the mutations and accumulation of p53 in MCF-7/adr cells (39). Additionally, NF-κB showed a considerably higher expression in the MCF-7/adr cells. Because of the known involvement of both NF-κB and HSF-1 in the regulation of the MDR1 gene, we sought to determine the expression of
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P-gp. Indeed, Western blot analysis of P-gp showed high levels in MCF-7/adr cells, whereas in MCF-7 cells, there was no P-gp observed (Fig. 1A).

RT-PCR indicated that the observed changes in expression of both HSF-1 and P-gp induced by chronic Dox exposure were due to changes in transcription (Fig. 1C). EMSA experiments showed no HSF-1 binding to HSE consensus oligonucleotides in nuclear extracts from MCF-7/adr cells, confirming that the observed decrease in HSF-1 protein in MCF-7/adr cells was accompanied by a decrease in functional HSF-1 (Fig. 1B). Thus, we conclude that inhibition of HSF-1 transcriptional activity produces the observed abrogation of Hsp27 expression in MCF-7/adr cells. These results establish that accumulation of mutp53 suppresses constitutive expression of HSF-1 (or vice versa) and Hsp27, resulting in higher expression of NF-κB and P-gp expression (Fig. 1A), as further supported below. To prove the causal relationship between NF-κB, HSF-1, and MDR1, additional experiments with a reporter gene assay were carried out. MDR1 promoter fragment was generated as described before (21, 22). MDR1 promoter has both CAAT (−116 to −113 bp; a putative NF-κB binding site) and GAA- CATTAC (−175 to −167 bp; an HSF-1 binding motif) (21, 22) sequences, indicating that both NF-κB and HSF-1 can bind to the promoter. Two MDR1 promoter fragments, the whole sequence (241 bp) and an HSF-1 binding site motif-deleted promoter fragment (200 bp), were cloned into the pGL3B luciferase vector, and luciferase activity was determined in MCF-7 and MCF-7/adr cells (Fig. 1D). MCF-7/adr cells showed, expectedly, about 10-fold higher luminescence than MCF-7 cells. Interestingly, in MCF-7 cells, deletion of the HSF-1 motif enhanced the luminescence, whereas the MCF-7/adr cells did not significantly enhance the luminescence, indicating that in MCF-7 cells, HSF-1 binding does inhibit the MDRI gene expression.

Treatment with Dox induced wtP53 and Hsp27 expression in MCF-7 cells, whereas no significant change was observed in their levels in MCF-7/adr cells. Dox transiently induced wtP53, which peaked at 12 h and decayed, as described in the quantitative plot (Fig. 2, A and B), whereas Hsp27 showed a very slight increase from basal level and remained at the same level. These results are consistent with our previous report that wtP53 undergoes Hsp27-assisted proteasomal degradation in UV-induced DNA-damaged MCF-7 cells (40). Although wtP53 and mutP53 were indistinguishable by Western blotting because the antibody recognizes both, mutP53 is found to be very stable, unlike wtP53. Thus, the transient appearance and disappearance after 48 h in MCF-7 is attributed as wtP53, whereas the strong prolonged existence is attributed as mutP53. However, neither induction of Hsp27 nor decay of mutP53 was observed in MCF-7/adr cells post-drug treatment (Fig. 2A), probably due to silencing HSF-1 produced by chronic exposure to Dox and the subsequent lack of Hsp27 in these cells. In order to confirm that HSF-1 is not activated during the Dox treatment in MCF-7/adr cells, EMSA experiments were carried out (Fig. 2C). There was only a minimal level of HSF-1 binding on HSE consensus sequence oligonucleotides detected upon treatment with Dox in MCF-7/adr cells, with no apparent increase with time, whereas DNA binding was significant and increased with post-drug treatment time points in MCF-7 cells (Fig. 2C). Supershift and antibody competition confirmed that HSF binding is due to HSF-1 (Fig. 2D, column 6). The protein expression profiles, obtained from Western blotting, revealed that the cell death occurred by the usual and established pathway in MCF-7, typically induction of Cdc2 kinase inhibitor p21, G2/M cell...
cycle arrest (as confirmed by flow cytometry; supplemental Fig. 1B), higher BCl-2, etc. (supplemental Fig. 1A). However, no evidence of such a G2/M cell cycle arrest pathway was noticed in MCF-7/adr cells (supplemental Fig. 1A and B). These results once again confirm that suppression of HSF-1 is correlative to the mutant p53 accumulation and leads to P-gp establishment through unknown mechanism in MCF-7/adr cells.

**Hsp27 Overexpression Depletes Mutant p53, Sensitizes MCF-7/adr Cells to Dox, and Enhances Cell Death**—MCF-7 and MCF-7/adr cells were transiently transfected with pCMV-SPORT6-Hsp27 plasmid. Hsp27 overexpression was very robust (>100-fold increase) in MCF-7/adr cells compared with pCMV-SPORT6 (control plasmid without Hsp27 cDNA)-transfected cells (Fig. 3A), producing Hsp27 levels comparable with the parental MCF-7 cells under basal conditions (Fig. 3B). However, transfection of MCF-7 with pCMV-SPORT6-Hsp27 showed a moderate increase in Hsp27 expression (<5-fold increase) (Fig. 3B). Hsp27-overexpressing MCF-7/adr (MCF-7/adr/Hsp27) and control MCF-7/adr cells were treated with increasing concentrations of Dox. MCF-7/adr/Hsp27 showed a significantly higher rate of cell death at all concentrations of Dox tested, where the IC50 was reduced to 4.1 µg/ml (Fig. 3C). However, control plasmid-transfected cells showed only 18% cell death, close to untransfected MCF-7/adr cells. Hsp27 transfected MCF-7 cells did not show any significant change in cell death (supplemental Fig. 2).

To determine whether the increased cell death in MCF-7/adr/Hsp27 cells is related to mutp53 depletion, as observed for the parental MCF-7 cells (Fig. 2A), the p53 level was determined. Upon treatment with increasing concentrations of Dox, the mutp53 level progressively decreased in MCF-7/adr/Hsp27 cells (Fig. 4A), similar to the decrease observed in the Dox-sensitive MCF-7 cells. Whereas there was a small increase in total Hsp27, observed at increasing Dox concentrations, there...
was a significant increase in the phosphorylation of Hsp27 (Ser-15) as Dox concentration increased. Hsp27 is known to be phosphorylated by MAPKAP-2, a downstream target of p38 MAPK (41), and Hsp27 phosphorylation increased only upon treatment with an increased concentration of Dox (Fig. 4A), indicating higher MAPK activity in the Dox-treated MCF-7/adr/Hsp27 cells. Indeed, the active phosphorylated p38 MAPK form was increased upon treatment with Dox in MCF-7/adr/Hsp27 cells, with no change in MCF-7/adr cells. Interestingly, the level of total p38 MAPK was not altered in both conditions studied (Fig. 4A). Similarly, the phospho-MAPKAP-2 was noted only in Dox-treated MCF-7/adr/Hsp27 cells and not in MCF-7/adr cells (Fig. 4A). The quantitative analysis showed that the p53 decreased more than three-fourth in the Dox concentration range 0–10 μg/ml (Fig. 4B), whereas Hsp27 phosphorylation increased about 4-fold over the same increase in Dox concentration (Fig. 4B). These results establish that forced expression of Hsp27 in MCF-7/adr cells enhances degradation of p53 and p38 MAPK activity upon treatment with Dox.

**Hsp27 Overexpression Represses the NF-κB Activity in MCF-7/adr Cells**—Canonical activation of NF-κB p65/p50 heterodimer by the IKK complex has been shown to trigger antiapoptotic pathways (42), whereas increased nuclear trans-localization of the p50 (NF-κB1) homodimer is considered as a negative feedback to regulate NF-κB activity (43). As shown in Fig. 1A, the NF-κB level was very high in MCF-7/adr cells, correlating to the observed higher P-gp level (Fig. 1A). The effect of Hsp27 overexpression on NF-κB activity in MCF-7/adr cells is illustrated in Fig. 5. Representative Western blots of p50 precursor (105 kDa), p50, and p65 (RelA) in cytosolic and nuclear extracts from MCF-7/adr and MCF-7/adr/Hsp27 cells that were treated with various concentrations of Dox are shown in Fig. 5A. As expected, the precursor of p50 (105-kDa protein) is localized in the cytoplasmic fraction, and there was not much change in the level of p50 precursor upon treatment with Dox in both MCF-7/adr and MCF-7/adr/Hsp27. However, the p50 level in nuclear extracts of MCF-7/adr/Hsp27 cells showed an increasing trend with an increase in Dox concentration (Fig. 5A). Conversely, the p65 level is reduced in the nuclear fraction of Dox-treated MCF-7/adr/Hsp27, illustrating a reverse trend to p50. The phosphorylated p65 also showed significantly reduced levels in MCF-7/adr/Hsp27 cells when compared with MCF-7/adr cells (Fig. 5A). The quantitative data presented as the ratio of p65/p50 (Fig. 5B) for nuclear fractions showed that the ratio decreased with an increase in Dox concentration in MCF-7/adr/Hsp27 cells, suggesting that transcriptional activation by canonical NF-κB activation is increasingly inhibited in MCF-7/adr/Hsp27 with Dox concentration. Confocal microscopic fluorescent imaging of p65 also confirmed this trend (Fig. 5C). Hsp27 has been found to interact with IKKα and regulate its activity (44). Thus, we then determined IκBα and IKKα in these cells. The inhibitor of NF-κB, namely IκBα, was found to decrease in Dox-treated MCF-7/adr/Hsp27 cells (Fig. 5A). Also, the IKKα was found to moderately increase upon treatment with Dox in these cells (Fig. 5A). These results clearly demonstrate that overexpression of Hsp27 attenuates the level of NF-κB expression, which is known to be transcriptionally repressive for MDR1 gene expression, and attenuates NF-κB transcriptional activity in MCF-7/adr cells.

**Hsp27 Overexpression Attenuates P-gp Expression and Retains Higher Intracellular Dox in MCF-7/adr Cells**—Western blots of P-gp in MCF-7/adr and MCF-7/adr/Hsp27 cells, treated with various Dox concentrations, are shown in Fig. 6A. Upon treatment with Dox, ectopic expression of Hsp27 suppressed the expression of P-gp, as seen in reduced protein levels in Western blots of 5- and 10-μg Dox-treated cells (Fig. 6A). The mRNA levels of P-gp, determined by RT-PCR, were consistent with the protein expression level as shown in Fig. 6B.
suggested that MDR1/P-gp expression is susceptible to down-regulation by the combination of Hsp27 overexpression and Dox treatment. Next, we determined the functional correlation of P-gp. To test the functionality of P-gp, in the presence of overexpressed Hsp27, we measured Dox accumulation by using confocal microscopic images in MCF-7/adr and MCF-7/adr/Hsp27 cells. Hsp27-overexpressing cells retained more Dox than MCF-7/adr cells (Fig. 6C). To quantitatively demonstrate the Dox uptake, efflux, and modulation of multidrug resistance in MCF-7/adr cancer cells, flow cytometry was carried out. The data presented in Fig. 6D show a concentration-dependent increase in intracellular fluorescence of Dox in MCF-7/adr/Hsp27 cells compared with the MCF-7/adr cells. Collectively, these results indicate that in MCF-7/adr cells Dox efflux occurred almost completely, whereas in Hsp27-overexpressing cells, about 40% of the cells retained Dox with average fluorescence intensity shown in Fig. 6D, which is consistent with the observed down-regulation of P-gp in Hsp27-overexpressing cells. We performed additional flow cytometry experiments with an unrelated dye to further confirm that P-gp function is reduced in Hsp27-overexpressing MCF-7/adr cells. Cell tracker blue dye (CMAC), which has 330- and 450-nm excitation/emission wavelengths that are out of the Dox excitation/emission range, was used to stain these cells and analyzed using flow cytometry. The results showed that the fluorescence was higher in Hsp27-overexpressing MCF-7/adr cells than in the MCF-7/adr cells (Fig. 6E), indicating that CMAC dye was retained more in the MCF-7/adr/Hsp27 cells than in the MCF-7/adr cells.

Hsp27 Overexpression Reestablishes Dox-induced Cytotoxicity and Apoptotic Pathway in MCF-7/adr Cells—Because Hsp27 overexpression led to the retention of Dox in MCF-7/adr cells, further experiments were carried out to determine the mechanism of cell death by Dox in MCF-7/adr/Hsp27 cells. As shown in Fig. 7A, Dox treatment of MCF-7/adr/Hsp27 cells led to a pronounced accumulation of the Cdc2 kinase inhibitor p21, whereas there was no detectable level of p21 in Dox-treated MCF-7/adr cells in the same Dox concentration range. Furthermore, Hsp27 overexpression decreased BCI-2 protein content in MCF-7/adr cells in a Dox concentration-dependent manner, and the apoptotic index (Bax/Bcl2 ratio) is increased in MCF-7/adr/Hsp27 cells.

Because the p21 was found to be selectively increased in MCF-7/adr/Hsp27 cells and it has been established that p21 induces cell cycle arrest, we next studied the effect of Dox on distribution of different phases of cell cycle using flow cytometry. Fig. 7B shows histograms of DNA contents of both MCF-7/adr and MCF-7/adr/Hsp27 cells that were treated with 1–10 μg/ml Dox. In MCF-7/adr cells (where very low levels of Hsp27 are expressed), Dox did not significantly change the cell population distribution in the G1, G2, and S phases, even up to 10 μg/ml Dox at the 48-h post-drug treatment time point (Fig. 7B). However, consistent with the observed increase in p21, in MCF-7/adr/Hsp27 cells, Dox induced G2/M arrest, the usual mechanism by which Dox normally induces cell death (45). Although p21 has been generally found to arrest G1/S phase, in the case of Dox, previous studies have shown that induction of p21 led to prolonged G2/M phase cell cycle arrest and apoptosis (46, 47). The quantitative G2/G1 ratio data, as presented in Fig. 7C, clearly demonstrate that the G2 population is more than 2.5 times greater in MCF-7/adr/Hsp27 cells. Increased cell death and cell cycle arrest upon Hsp27 overexpression indicate that...
these cells exhibit an apoptotic pathway rather than DNA repair and survival.

To substantiate that the MCF-7/adr/Hsp27 cells undergo increased apoptosis upon treatment with Dox, through a p53-dependent intrinsic apoptotic pathway, PARP-1 integrity was determined. It has been very well established that caspase-3/7 activation leads to proteolysis of the DNA repair enzyme PARP-1, which can be detected by Western blot by the appearance of a distinct band at 89 kDa indicating cleaved PARP-1 (48). Dox treatment of MCF-7/adr/Hsp27 showed a cleaved PARP-1 band at 89 kDa, which increased upon increasing Dox concentration. Conversely, Dox-treated MCF-7/adr cells did not show any discernable PARP cleavage (Fig. 8A). Although caspase-3 was observed to decline in Hsp27-overexpressing MCF-7/adr (Fig. 8A), the cleaved caspase-3 could not be detected. Thus, we analyzed caspase-7 and found a distinct 28-kDa cleaved product in Dox-treated Hsp27-overexpressing MCF-7/adr cells (Fig. 8A). Thus, our results indicate that the apoptosis by PARP-1 cleavage is via the caspase-9/7 pathway. Taken together, these data suggest that the apoptosis in MCF-7/adr/Hsp27 cells is mediated by the canonical caspase-7/9-dependent pathway.

**DISCUSSION**

The present work has revealed that chronic exposure of the human breast cancer cell line, MCF-7, to Dox inhibits HSF-1 expression and its transactivation, leading to decreased Hsp27. The absence of Hsp27, which is required for p53 proteasomal degradation by ubiquitination (39), results in accumulation of mutp53, and that in turn triggers the NF-κB-dependent signaling pathway to induce MDRI gene and P-gp efflux pumps in MCF-7/adr cells. Although various aspects of the MDRI/P-gp induction mechanism have been studied previously in MCF-7 cells (49), for the first time we report here that endogenous silencing of HSF-1 by mutp53 and inhibition of Hsp27 protein expression is an important step in the MDRI expression and in establishing P-gp transporters, with the help of higher NF-κB activity. The present work has also shown that Hsp27 overexpression reverses P-gp-induced drug efflux in the MCF-7/adr cells. Multidrug resistance is established by inducing various drug efflux mechanisms involving ABC transporters, such as P-gp, MRP1, and BCRP. Interestingly it was found in the MCF-7/adr cells that there is a complete inhibition of Hsp27 expression (no trace in Western blots; Fig. 1A), whereas there was large increase in mutant p53 at basal and resting conditions (Fig. 1A). This is contrary to the general consensus that Hsp27 overexpression induces drug resistance in different cancer cells (30, 31, 33–36). Previous studies have shown that HSF-1 overexpression induces drug resistance (29, 50). However, these studies were carried out in drug-sensitive cells. Our study is the first to evaluate the functional relationship between MDRI and HSF-1 in drug-resistant cells.

Indeed, quantity of inducible Hsp27 is reportedly one of the pivotal conditions in determining cellular resistance to a variety of stress factors, including chemotherapeutic drug resistance in cells (51), and it is strictly dependent on stress-regulated activation of its transcription factor HSF-1. This mechanism has repeatedly been shown to enable cytoprotection and cell survival. For example, induction of Hsps, including Hsp27, in MCF-7 and MDA-MB-231 breast cancer cells following heat shock treatment resulted in specific resistance to Dox but not colchicines, cisplatin, and actinomycin D (2). Selective overexpression of Hsp27 in MDA-MB-231 and Chinese hamster ovary cells was also shown to induce resistance to Dox (52). All of these studies were, however, carried out on Dox-sensitive cells, where Dox can induce damage-exerting cytotoxicity, and that toxicity is opposed by Hsp27. Contrarily, despite high level resistance to Dox in MCF-7/adr cells, they express an almost undetectable level of Hsp27 (Fig. 2A). The difference in the role of Hsp27 in drug-sensitive and drug-resistant cells suggests that Hsp27 may play an important role in the development of drug resistance.

**FIGURE 7. Higher Dox-induced cytotoxicity in MCF-7/adr/Hsp27 cells.** The cells were treated with different concentrations of Dox as indicated in the figure, and after 48 h, the cells were fixed in ethanol and subjected to flow cytometry. A, Western blots of p21, Bcl-2, and Bax in these cells, treated with different concentrations of Dox as indicated. B, flow cytometry analysis of cell cycle arrest induced by overexpressed Hsp27 in MCF-7/adr/Hsp27 cells. C, quantitative plots of G2/G1 ratio, determined from three independent measurements. Error bars, S.E.
of Hsp27 relies on its influence on p53 degradation. In sensitive cells, Hsp27 has been reported to inhibit apoptosis in multiple ways. First, it has been found to accelerate wtp53 degradation so that p53-dependent apoptosis could be suppressed. Also, Hsp27 has been reported to be directly involved in caspase inhibition (53). In MCF-7/adr, most p53 expressed is mutant (P72A and deletion of 126–133 residues) (22) with gain of function that can transcriptionally repress many proteins, including HSF-1, as found in the present work. Thus, it appears that inhibition of transcriptional activation of HSF-1 and Hsp27 expression, either by accumulated mutant p53 or some other unknown pathway, is responsible at least in part for expression of MDR1/P-gp in MCF-7/adr cells. This interpretation is consistent with the report that overexpression of mutant HSF-1 (deletion of 202–312 residues), the domain containing leucine zippers that is required for trimerization and activation) induced MDR1 in HeLa cells (29).

Although HSF-1 is considered to be the transcription factor responsible for Hsp27 induction, other factors can also induce Hsp27 (54). For example, wild type p53, a proapoptotic protein, was reported to induce Hsp27 expression in prostate cancer cells but not by mutant p53. This is consistent with present findings in MCF-7/adr cells that despite significant mutant p53 observed in MCF-7/adr cells, there is no Hsp27 detected (Fig. 1A). Interestingly, there was no HSF-1 either. Thus, it appears that loss of Hsp27 and mutation of p53 operates synergistically toward the adaptation of these cells against Dox, inducing the P-gp drug efflux pumps. However, the exact mechanism explaining how mutp53 can transcriptionally regulate such synergistic pathways is not currently known. Although wtp53 DNA binding properties have been documented (55), mutp53 DNA binding is still largely unknown (56). First, the HSF-1 promoter does not have any consensus sequence motifs for wtp53 binding, whereas the Hsp27 promoter does. Thus, wtp53 cannot inhibit or activate HSF-1 gene transcription as such. The mutp53 observed in MCF-7/adr cells shows a different mechanism of DNA binding. The mutation found in mutp53 of MCF-7/adr is primarily in the sequence-specific DNA binding domain (p53SSBD), whereas the C-terminal domain (p53CTD) DNA binding domain is still intact. It is known that p53 can bind to DNA by both p53SSBD and p53CTD. p53SSBD requires a specific DNA target sequence, whereas p53CTD binding is based on the recognition of target DNA by structural features rather than sequence motif (57). In addition, p53CTD can associate with other transcription factors forming facultative complexes that can bind to the respective transcription factor binding sites to modulate transcription properties (57). Because mutp53 has an intact CTD, mutp53 is still able to silence the HSF-1 gene through an unknown mechanism, by recognizing structurally flexible and non-linear DNA structures. Furthermore, it has been proposed that CTG-CAG repeats are required for such binding (57). Although HSF-1 has many such repeat units in the promoter region, it is not certain whether mutp53 represses the HSF-1 by direct binding or by forming facultative complexes with other transcription factors in MCF-7/adr cells.

Direct transcriptional regulation of the MDR1 gene by wtp53 and mutp53 has been suggested previously (58). It has been reported in human colon carcinoma cells that mutant p53 induces endogenous MDR1 (59). However, the relationship between MDR1 and p53 is conditional (i.e. it is dependent on the cellular environment and the drug being used). It has been proposed that wtp53 generally represses the MDR1 promoter through interaction with basal transcription factors, such as TATA-binding protein (24). However, this seems unlikely, given that mutants of p53 in the C & N terminus are still capable of interacting with TATA-binding protein but are unable to repress transcription (25, 26). Indeed, the MDR1 promoter has been characterized to have a wtp53 consensus binding site, and it appears that p53 binding at this site results in repression of MDR1. Because mutp53 can promote MDR1 by direct interaction with the MDR1 gene, transcriptional regulation of MDR1 by mutp53 could be based on p53CTD binding to structural specific DNA as discussed above for HSF-1 (57). Hence, the only way to repress MDR1 and reduce P-gp is removal of mutp53. Previous reports have shown that pharmacological agents could successfully repress MDR1 gene transcription (60). The present work has not only identified inhibition of
NF-κB in these cells, mutant p53 accumulation and higher NF-κB activity is able to overcome P-gp in MCF-7/adr cells. Moreover, based on efflux transporters are operative. Forced expression of p53 in MCF-7/adr cells that are known to repress NF-κB results. Further work is necessary to delineate their roles in the present work reveals only how the Hsp27, which is a potential mechanism for sensitizing multidrug-resistant MCF-7/adr cells to cause DNA damage and apoptosis. Thus, the absence of HSF-1 activity and induction of proapoptotic genes but also at the level of transcriptional activities of various other factors, such as p53 and RelA/p65, in antiapoptotic gene expression in MCF-7/adr cells. Therefore, modulators of HSF-1 and Hsp27 could serve as potential agents to circumvent multidrug resistance breast cancer treatment in clinic.

In summary, in the present work, we have demonstrated a novel mechanism of sensitizing multidrug-resistant MCF-7/adr cells through overexpression of Hsp27 (Fig. 8B). Although Hsp27 overexpression has been generally reported to produce an antiapoptotic phenotype and drug resistance and the use of Hsp27 siRNA has been proposed as a potential combination therapy with chemotherapeutics for improved outcome in different malignantities (38), we have demonstrated here that HSF-1 and Hsp27 depletion is observed when MDRI and P-gp-based efflux transporters are operative. Forced expression of Hsp27 is able to overcome P-gp in MCF-7/adr cells. Moreover, in these cells, mutant p53 accumulation and higher NF-κB activity are also observed. Upon overexpression of Hsp27 by transfection and Dox treatment, these pathways are suppressed, P-gp level is significantly reduced, and the Dox is retained in MCF-7/adr cells to cause DNA damage and apoptosis. Thus, our findings suggest that Hsp27 overexpression dictates cell fate decisions not only at the level of cell cycle arrest and induction of proapoptotic genes but also at the level of transcriptional activities of various other factors, such as p53 and RelA/p65, in antiapoptotic gene expression in MCF-7/adr cells. Therefore, modulators of HSF-1 and Hsp27 could serve as potential agents to circumvent multidrug resistance breast cancer treatment in clinic.

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HSF-1 Inhibition Induces MDRI Gene in MCF-7 Cells

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