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Apoptosis, autophagy, accelerated senescence and reactive oxygen in the response of human breast tumor cells to Adriamycin

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ABSTRACT

Although the primary response to Adriamycin (doxorubicin) in p53 mutant MDA-MB231 and p53 null MCF-7/E6 breast tumor cells is apoptotic cell death, the residual surviving population appears to be in a state of senescence, based on cell morphology, beta galactosidase staining, induction of $p21^{waf1/cip1}$ and down regulation of cdc2/cdk1. Suppression of apoptosis in MDA-MB231 and MCF-7/E6 cells treated with Adriamycin using the broad spectrum caspase inhibitor, zvad-Fmk, results in substantial induction of autophagy. Overall sensitivity to Adriamycin, measured by clonogenic survival, is not altered in the cells undergoing autophagy, consistent with autophagy contributing to cell death in response to Adriamycin. The free radical scavengers, glutathione and N-acetyl cysteine attenuate the accelerated senescence response to Adriamycin in MCF-7 cells as well as in MDA-MB231 and MCF-7/E6 cells, but protect primarily the MCF-7 cells, indicating that reactive oxygen is unlikely to be directly responsible for Adriamycin toxicity in breast tumor cells. Expression of caspase 3 or induced expression of c-myc in MCF-7 cells fails to abrogate accelerated senescence induced by Adriamycin. Taken together, these studies suggest that accelerated senescence induced by Adriamycin is similar in cells with wild type p53 and in cells lacking functional p53 with regard to the upregulation of $p21^{waf1/cip1}$, down regulation of cdc2 and the involvement of reactive oxygen species. Furthermore, accelerated senescence, autophagy and apoptosis all appear to be effective in suppressing self-renewal capacity in breast tumor cells exposed to Adriamycin.

1. Introduction

It is becoming increasingly apparent that tumor cells have the capacity to respond to chemotherapy and radiation through multiple growth arrest and cell death pathways [1,2]. Cell death in leukemia and lymphoma derived tumor cells frequently occurs through apoptosis [3,4], while mitotic catastrophe is a well established response to ionizing radiation [5]; in addition,

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Abbreviations: ADR, Adriamycin (doxorubicin); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; GSH, glutathione; NAC, N-acetyl cysteine.

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autophagy or type II apoptosis is recognized as having the potential to contribute to cell killing in addition to its known cytoprotective function associated with nutrient deprivation [6,7]. Self-renewal capacity in the tumor cell can also be abolished through accelerated or premature senescence, a form of growth arrest that has been observed primarily, although not exclusively, after exposure to ionizing radiation or drugs that induce DNA damage [8,9]. Like replicative senescence, cells undergoing accelerated senescence are characterized by enlargement, flattening, granulation and expression of a pH 6.0 dependent beta galactosidase activity [8–10]. Accelerated or premature senescence has also been identified in tumor cell xenografts exposed to chemotherapeutic agents [2], has been shown to mediate tumor regression [11,12], in part through the involvement of the immune system, and has been reported in clinical tumor samples in patients receiving chemotherapy [13,14].

The signaling pathway that promotes accelerated senescence overlaps, in large part, with that for conventional growth arrest in terms of the induction of p53 [15] and the cyclin dependent kinase inhibitory protein, $p21^{waf1/cip1}$ [15] as well as down regulation of cdc2/cdk1 [9,13,16,17]; however, there is also evidence for accelerated senescence that is independent of p53 [13,18]. While the induction of senescence does not appear to require functional p16 [18–21], p16 may be critical for maintenance of the senescence-arrested state [22,23]. Suppression of c-myc has been shown to promote senescence [24–26], an observation which may be related to the capacity of both p53 and $p21^{waf1/cip1}$ to suppress transcription of c-myc [27,28]. Finally, mitochondrial reactive oxygen generated downstream of $p21^{waf1/cip1}$ has been implicated in replicative senescence [29], while the role of reactive oxygen, if any, in accelerated senescence awaits definition.

In view of the fact that tumor cells can respond to stresses such as radiation and chemotherapeutic drugs through alternative cell death pathways when the ''primary'' pathway is compromised or attenuated [30–35], we were interested in assessing whether multiple mode(s) of cell death (and/or growth arrest) mediate the response to the chemotherapeutic drug, Adriamycin, in breast tumor cells that have been shown to be highly apoptosis competent (cells mutant or null in p53 as well as p53 wild type MCF-7 cells expressing caspase 3 [20,33]). We also further addressed the signaling pathways involved in the accelerated senescence response to Adriamycin, focusing on the induction of $p21^{waf1/cip1}$, the down regulation of cdc2 and c-myc and the potential contribution of reactive oxygen species.

2. Materials and methods

2.1. Materials

RPMI 1640 medium with L-glutamine, trypsin-EDTA (1×; 0.05% trypsin, 0.53 mM EDTA-4 Na), penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin), and fetal bovine serum were obtained from Invitrogen (Eugene, OR). Defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). Reagents used for the TUNEL assay (terminal transferase, reaction buffer, and Fluorescein-dUTP) were purchased from Roche Diagnostics Corporation (Germany). X-gal was obtained from Gold Biotechnology (St. Louis, MO). The following materials were obtained from Sigma Chemical (St. Louis, MO): formaldehyde, acetic acid, albumin bovine (BSA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), monodansylcadaverine (MDC), N-acetyl-L-cysteine (NAC), reduced glutathione (GSH), 6-diamidino-2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO). Acridine orange was purchased from Molecular Probes (Eugene, OR). zVAD-fmk was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Adriamycin was obtained from Sigma Chemical Company, St. Louis, MO, reconstituted in molecular biology grade water, and stored as aliquots at −20 °C until dilution in culture media for treatments. Antibodies for p53 and $p21^{WAF-1}$ were purchased from Signal Transduction Laboratories), cdc-2 from Santa Cruz Biotechnology, anti-mouse IgG from KPL Inc. and β-actin from Sigma Chemical Company. Reporter constructs for the luciferase assay were purchased from Addgene Inc. (Cambridge, MA). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI).

2.2. Cell culture and treatment regimens

The MCF-7 breast tumor cell line was obtained from the NCI Frederick Cancer Research Facility. The isogenic cell line, MCF-7/E6, was established by stable retroviral infection as described previously [20]. MCF-7/35im cells with doxycycline-inducable myc were established as described previously [36]. The MCF-7 cells expressing caspase 3 were described in previous studies [33]. Cells were maintained as monolayer cultures in RPMI 1640 media supplemented with glutamine (0.292 mg/ml), penicillin/streptomycin (0.5 ml/100 ml media), and 10% fetal bovine serum. Cells were cultured at 37 °C in 5% CO_2 and 100% humidity. 24 h after plating, cells were exposed to either 0.75 μM or 1 μM Adriamycin for 2 h. Cells were washed free of drug and cultured in fresh media for the subsequent period of the experimental protocols.

2.3. Effects on GASH and NAC on sensitivity to Adriamycin by the MTT assay

To determine the effects of GSH or NAC on sensitivity to Adriamycin in MCF-7, MDA-MB231 and MCF-7/E6 cells, cells were seeded in triplicate wells at 8000 cells per well of a 96-well cluster plate and were treated with 1 μM of Adriamycin for 2 h in the presence or absence of 20 mM GSH or 20 mM NAC, followed by removal of the drugs and washing of the cells. At 72 h post-drug exposure, cell viability was assessed using a standard MTT assay. This involved adding 100 μL of 2 μg/mL MTT per well, incubating in the dark for 3 h, carefully removing the MTT, and then adding 100 μL DMSO per well. Absorbance was measured at 490 nm with an EL800 Universal Microplate Reader (Bio-Tek Instruments Inc.).

2.4. Beta-galactosidase staining

Senescence-associated (SA) beta-galactosidase histochemical staining in the MDA-MB231 and MCF-7/E6 cells was performed as described previously [10,20] after exposure to 0.75 μM ADR. Cells were washed twice with PBS and fixed with 2%

formaldehyde, 0.2% glutaraldehyde for 5 min. The cells were washed again with PBS and stained with a solution of 1 mg/mL 5-bromo-4-chloro-3-inolyl-β-galactosidase in dimethylformamide (20 mg/mL stock), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0, and 2 mM $MgCl_2$. Following overnight incubation at 37 °C, the cells were washed twice with PBS and the percentage of stained cells was determined after counting three random fields of 100 cells each. Representative fields were photographed under a 10× objective.

2.5. TUNEL assay for apoptosis

The method of Gavrieli et al. [37] was utilized as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and non-adherent cells, as reported previously [20,33]. Cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using the In Situ Cell Death Detection Kit (Roche), where strand breaks are end labeled with fluorescein dUTP by the enzyme terminal transferase. TUNEL positive cells were quantitated by counting the number of positive cells per field using Image-Pro Plus software by Media Cybernetics, L.P. Three representative fields were averaged per condition.

2.6. Clonogenic survival assay

Cells were plated in triplicate in 6-well tissue culture dishes at the appropriate density for each condition and treated with Adriamycin, zVAD-fmk or Adriamycin plus zVAD-fmk. After 14 days, the cells were fixed with 100% methanol, air-dried for 1–2 days and stained with 0.1% crystal violet. For computing the survival fraction, groups of 50 or more cells were counted as colonies. Data were normalized relative to untreated controls that were taken as 100% survival.

2.7. Autophagy detection with acridine orange and monodansylcadaverine dye staining

As a marker of autophagy, the volume of the cellular acidic compartment was visualized by acridine orange staining, as described previously [33,38]. Cells were seeded in 6-well plate. After overnight incubation to allow for adherence, cells were exposed to 1.0 μM Adriamycin for 2 h. 48 h following treatment, cells were incubated with medium containing 1 μg/ml acridine orange (Molecular Probes, Eugene, OR) for 15 min. The acridine orange was removed and fluorescent micrographs were taken using an inverted fluorescent microscope. MDC (monodansylcadaverine) was used to confirm the abundance of autophagic vacuoles in cells. A 10 mM stock solution of MDC was prepared in 1:1 DMSO/EtOH immediately prior to its addition to cell cultures. Following Adriamycin treatment, cells were stained with 50 μM MDC for 10 min at 37 °C, washed with 1× PBS and examined by fluorescence microscopy.

2.8. Assessment of mitotic catastrophe

The induction of mitotic catastrophe was assessed by the formation of micronuclei in binucleated cells, as described previously [33,38]. Cells were seeded on glass cover slips and treated with ADR, zVAD-fmk or combination of ADR and zVAD-fmk. At the appropriate times post-treatment, cells on cover slips were fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Cells were then fixed to glass slides using DAPI-containing Vectashield mounting medium. Images were captured using a Zeiss laser scanning microscope.

2.9. Western blotting

Cell cultures were lysed in 60 mM Tris (pH 6.8) containing 2% SDS and a cocktail of protease inhibitors (Sigma Chemical Company) at the indicated time points. Whole cell lysates were boiled for 5 min, briefly sonicated, and then centrifuged for 10 min at 10,000 × *g* at 4 °C. Protein concentrations were determined using a Lowry-based spectrophotometric assay (BioRad, Hercules, CA), according to the manufacturer's protocol. 10–20 μg of each sample was separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. A standard blotting procedure was performed using monoclonal antibodies directed against p53 (1:5000), $p21^{WAF-1}$ (1:1000), c-myc (1:500; antibody was produced from the hybridoma cell line 9E10 in our laboratory) and cdc-2 (1:1000) followed by peroxidase-conjugated anti-mouse IgG (1:10,000). To control for protein loading, all membranes were subsequently probed with a β-actin antibody (1:2,000).

2.10. Luciferase assay

MDA-MB231 or MCF-7/E6 cells were co-transfected with a PG13 luciferase reporter construct containing promoter binding sites for wild type p53 or a MG-15 luciferase reporter construct containing promoter binding sites for mutant p53 along with a Renilla luciferase control reporter for 48 h. Six hour post-Adriamycin treatment (1.0 μM), cells were washed once with 2 ml of ice cold 1× PBS (pH 7.4) and then lysed in 200 μl of 1× lysis buffer provided with the Promega Dual-Luciferase Assay System. Luciferase activities were determined using 20 μL of the lysate/sample in a Turner Designs luminometer. The firefly luciferase/Renilla activity ratio reflected either wild type or mutant p53 activity.

2.11. Assessment of functional Adriamycin concentration in the presence and absence of GSH and NAC

Adriamycin was made up in phenol red free medium to a final concentration of either 1 μM or 10 μM and allowed to remain at room temperature either in the presence or absence of 10 mM glutathione or 10 mM N-acetyl cysteine (NAC). The fluorescence intensity of Adriamycin was measured spectrophotometrically at a wavelength of 485 nm (Ultrospec 3000, Pharmacia Biotech).

2.12. Statistical analysis

Statistical differences were determined using Statview statistical software. A one-way analysis of variance (ANOVA) followed by a Bonferroni/Dunn test was used to compare the difference between the Adriamycin treatment group and

the NAC or GSH plus Adriamycin groups in MCF-7/E6 or MDA-MB231 cells for the sensitivity studies in Fig. 8. All other comparisons were made using the paired Student's *t*-test. *P* values ≤ 0.05 were taken as statistically significant.

3. Results

3.1. *Senescence in breast tumor cells that lack functional p53*

Our previous studies comparing the response to Adriamycin in p53 wild type MCF-7 breast tumor cells and breast tumor cells with either mutant p53 (MDA-MB231) or p53 abrogated by the viral E6 protein (MCF-7/E6) strongly supported a requirement for functional p53 in promoting accelerated senescence [20]. In contrast to a prolonged senescent growth-arrested state in p53 wild type MCF-7 cells, Adriamycin was found to kill MDA-MB231 and MCF-7/E6 cells (primarily through apoptosis), leaving a small residual surviving cell population. In the studies presented in Fig. 1, we demonstrate that the cells surviving after acute exposure to 0.75 μM Adriamycin (for 2 h, followed by removal of the drug) were in a state of senescence, based on altered cell morphology and expression of a pH 6 dependent beta-galactosidase. Fig. 1 provides representative examples of senescent MDA-MB231 and MCF-7/E6 cells at 6 days after initiating exposure to Adriamycin, where the percent of the population that is senescent is indicated within the figure. The impact of treatment with zVAD-fmk is discussed in Section 3.4.

3.2. *p53-Independent induction of $p21^{waf1/cip1}$ and down regulation of cdc2*

The induction of $p21^{waf1/cip1}$ is a hallmark of both replicative and accelerated senescence [8,15]. Fig. 2 indicates that $p21^{waf1/cip1}$ was induced in MDA-MB231 and MCF-7/E6 cells exposed to Adriamycin (as well as in MCF-7 cells, utilized as a positive control). As expected, p53 was also induced in the MCF-7 cells but not in the MCF-7/E6 cells. A transient induction of mutant p53 in the MDA-MB231 cells is also evident.

Previous studies by this and other laboratories have suggested that down regulation of cdc2/cdk1 may be involved in the accelerated senescence response to chemotherapy [13,16,17,39,40]. Consistent with this premise, we observed down regulation of cdc2 in the senescing MCF-7/E6 and MDA-MB231 cells (as well as in MCF-7 cells, again used as a positive control), within 5 days after initiating drug exposure, as shown in Fig. 2.

The possibility that induction of $p21^{waf1/cip1}$ and the consequent promotion of senescence might be a consequence of residual wild type p53 activity in the MDA-MB231 or MCF-7/E6 cells was investigated with a luciferase reporter assay using constructs containing binding sites for either wild type or mutant p53 in the promoters linked to luciferase. Fig. 3 shows a slight upregulation of mutant p53 post-Adriamycin treatment in MDA-MB231 cells, which is consistent with the Western blotting data in Fig. 2. Both cell lines demonstrated evidence of some basal wild type p53 activity, but with no induction observed after treatment with Adriamycin. These studies strongly suggest that the induction of $p21^{waf1/cip1}$ by Adriamycin in MDA-MB231 and MCF-7/E6 cells occurred independently of p53 function.

3.3. *Senescence in breast tumor cells expressing caspase 3*

The detection of a surviving population of senescent MDA-MB231 and MCF-7/E6 cells after exposure to Adriamycin suggests that senescence is not simply a default response in cells that are incapable of an apoptotic response to drugs or chemotherapy, given that both MDA-MB231 cells and MCF-7/E6 cells are apoptosis competent [20]. In support of this

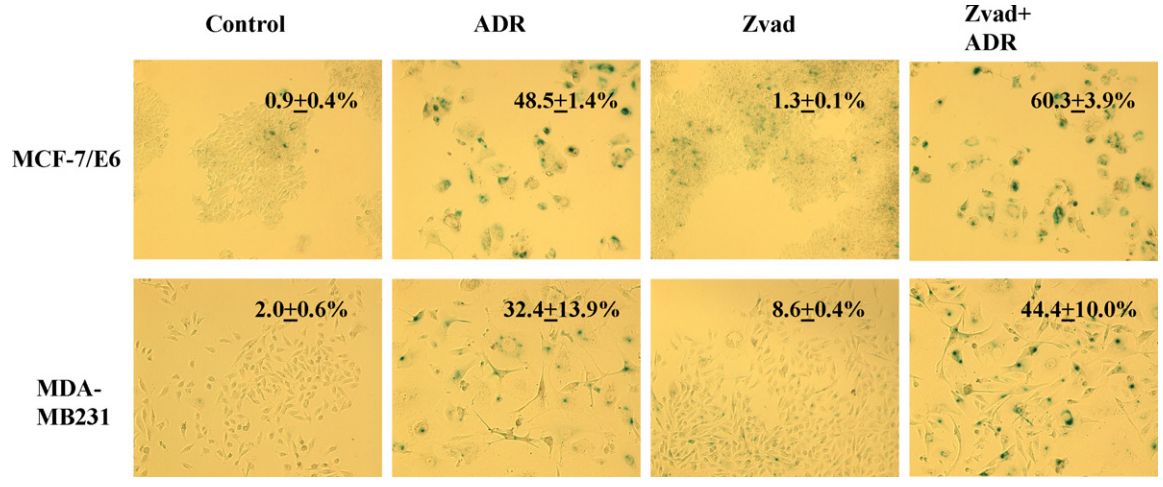

Fig. 1 – Senescence in residual surviving MDA-MB231 and MCF-7/E6 breast tumor cells. Cells were treated with 0.75 μM Adriamycin for 2 h with or without prior exposure to 50 μM zVAD-fmk for 1 h. Adriamycin was removed, the cells were maintained in the presence of the zVAD-fmk and senescence was assessed at day 6 after initiation of drug treatment. Senescence was detected based on alterations in cell morphology and expression of pH 6 dependent beta galactosidase activity. The percentage of the cell population in a senescent state is indicated within the figure. *P* values: 0.0237 for MCF-7/E6 + ADR compared to MCF-7/E6 + ADR + zVAD-fmk; *P* value: 0.4573 for MDA-MB231 + ADR compared to MDA-MB231 + ADR + Zvad-fmk; data is taken from three replicate experiments.

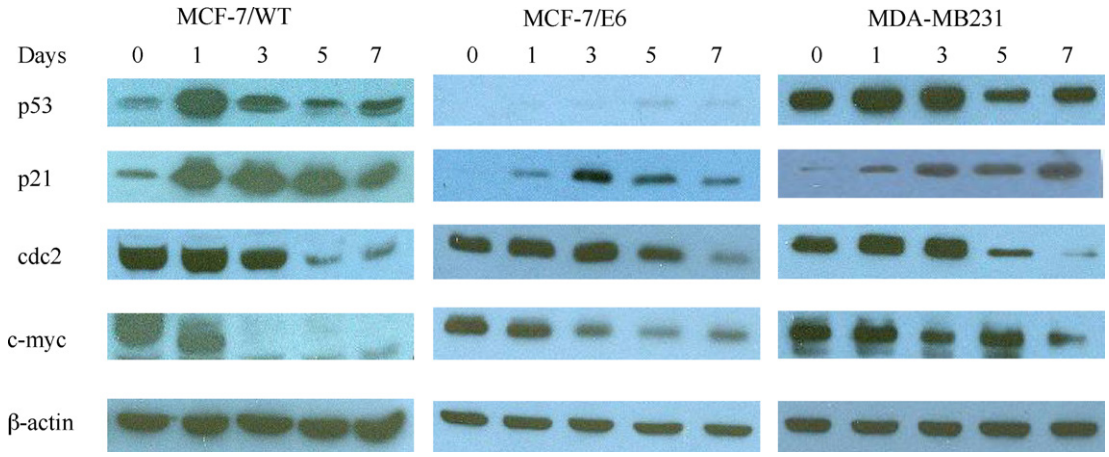

Fig. 2 – Alterations in selected proteins associated with the accelerated senescence response to Adriamycin. MCF-7, MDA-MB231 and MCF-7/E6 cells were exposed to 0.75 μM Adriamycin for 2 h and samples were isolated, electrophoresed and subjected to immunoblotting at the indicated times. Loading was controlled based on beta actin levels. The lanes demonstrating $p21^{waf1/cip1}$ induction in MDA-MB231 required a longer time of exposure (20 min compared to 30 s). Data are representative of three separate experiments.

contention, MCF-7 cells engineered to express caspase 3, the executioner caspase of apoptosis [41], also demonstrated an unequivocal senescence response to Adriamycin, albeit one that was slightly delayed in comparison to that in MCF-7 cells that do not express caspase 3. Fig. 4 shows the percentage of the senescent cell population detected in both MCF-7 and MCF-7/caspase 3 cells after exposure to Adriamycin as well as representative examples of the senescent cell populations.

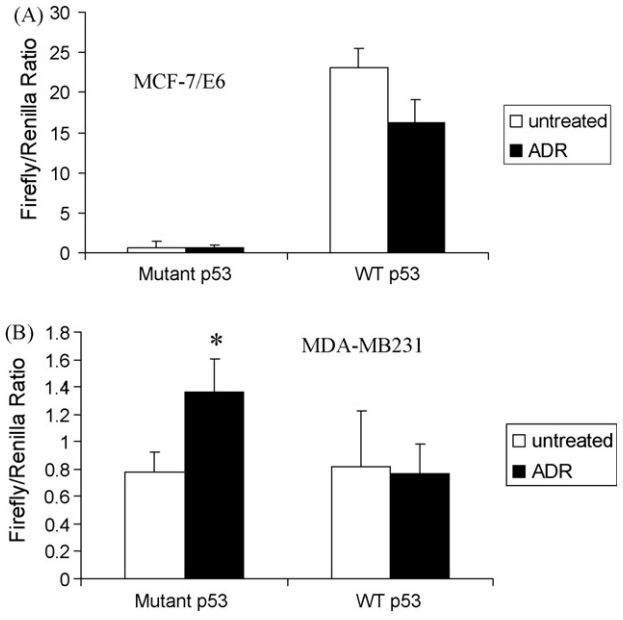

Fig. 3 – Luciferase Assay. MDA-MB231 and MCF-7/E6 cells were co-transfected with PG13-luciferase or MG-15 luciferase reporter constructs and a Renilla control reporter for 48 h followed by acute treatment with 1 μM Adriamycin. Firefly and Renilla luciferase activities were measured 6 h post-Adriamycin treatment by the Dual-Luciferase Reporter Assay System. (A) MCF-7/E6 cells; (B) MDA-MB231 cells.

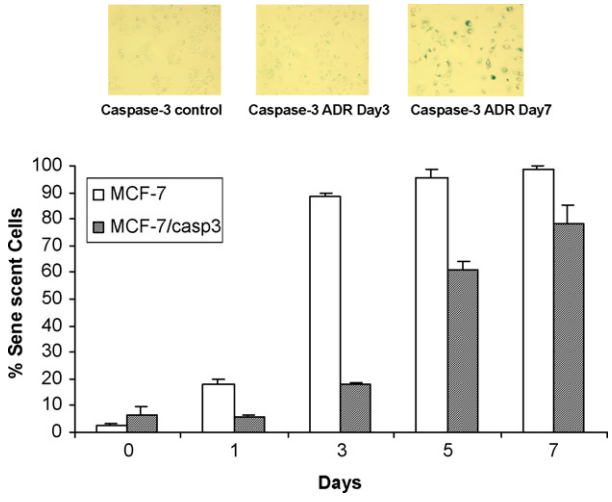

Fig. 4 – Senescence in MCF-7/caspase 3 cells. MCF-7 cells engineered to express caspase 3 were exposed to 0.75 μM Adriamycin for 2 h and senescence was monitored based on cell morphology and expression of beta galactosidase. Upper figure: images and beta galactosidase staining. Lower panel: time course quantitation of extent of senescence. Senescence in MCF-7 cells exposed to Adriamycin is presented as a positive control. Data is drawn from three separate experiments.

3.4. *A blockade to apoptosis promotes autophagy in MDA-MB231 and MCF-7/E6 cells*

It is becoming clear that there are likely to be multiple alternative pathways by which solid tumor cells can die (or abrogate self-renewal capacity) in response to stress. We were therefore interested in whether apoptosis and senescence might be reciprocally regulated responses to Adriamycin in breast tumor cells lacking functional p53. To this end, we examined whether interference with apoptosis in the MDA-

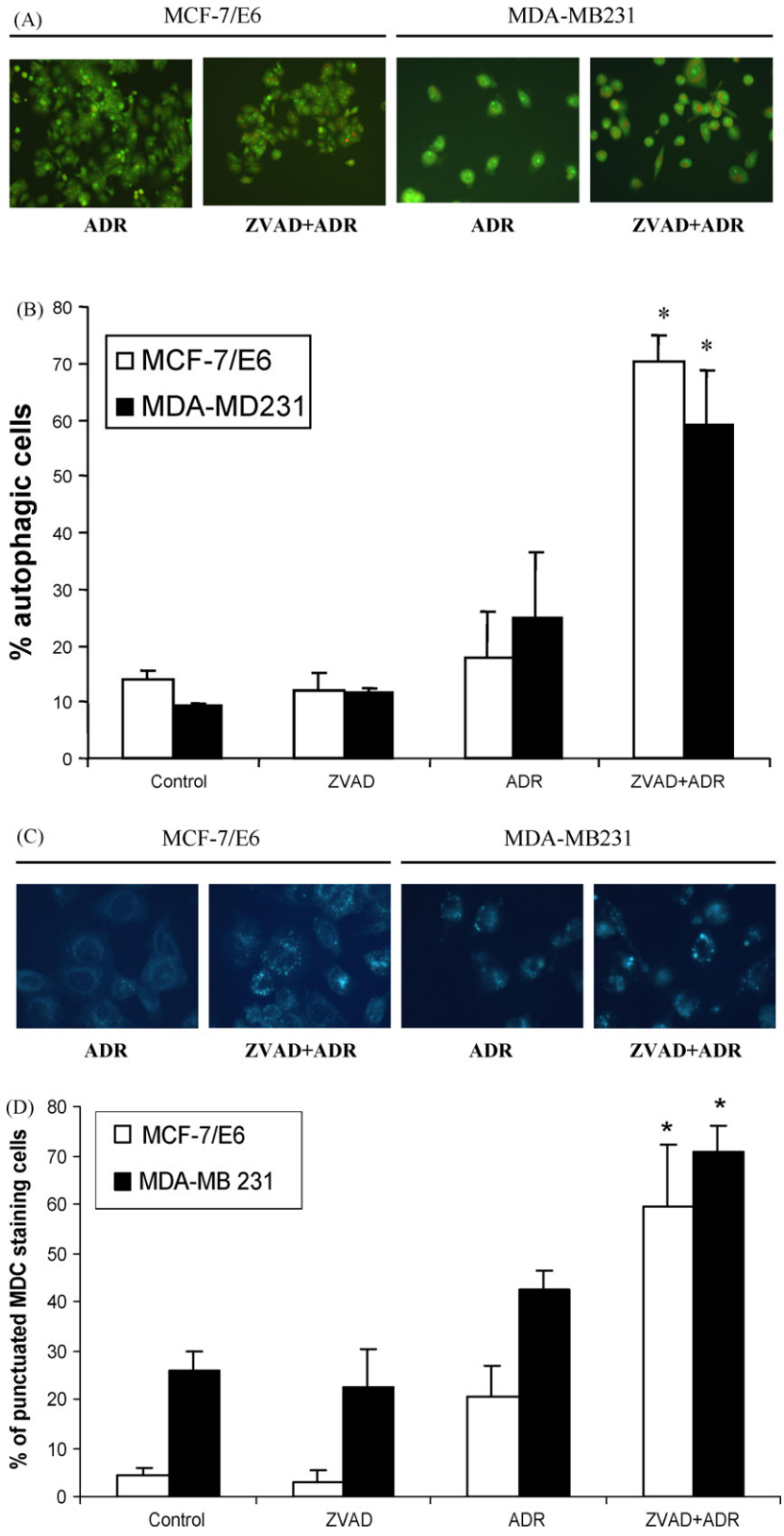

Fig. 5 – Autophagy in MDA-MB231 and MCF-7/E6 cells. MDA-MB231 and MCF-7/E6 cells that were pretreated with the broad spectrum caspase inhibitor, zVAD-fmk, for 2 h were exposed to 1 μM Adriamycin for 2 h and maintained in zVAD-fmk after Adriamycin was removed as in the studies in Fig. 1. Autophagy was assessed based on acridine orange and monodansylcadaverine dye staining 2 days after initiating exposure to Adriamycin. (A) Representative figures demonstrating autophagy by acridine orange dye staining in MDA-MB231 and MCF-7/E6 cells. (B) Quantification of the extent of autophagy in MDA-MB231 and MCF-7/E6 cells. Data presented is derived from three

MB231 and MCF-7/E6 cells would result in a pronounced enhancement of accelerated senescence.

Exposure to the broad spectrum caspase inhibitor, zVAD-fmk substantively blocked the apoptotic response in MDA-MB231 and MCF-7/E6 cells (a reduction of approximately 93% in MDA-MB231 cells and 75% in MCF-7/E6 cells; data not shown). As shown in Fig. 1, interference with apoptosis resulted in a small but significant increase in the extent of senescence in the MCF-7/E6 cells and a small (but statistically insignificant) increase in the extent of senescence in the MDA-MB231 cells. However, a blockade to apoptosis resulted in a substantial increase in the extent of autophagy in response to Adriamycin treatment in both cell lines. Representative images of acridine orange staining of autophagic vesicles on day 2 are shown in Fig. 5A. Quantification of the extent of autophagy is shown in Fig. 5B. Control cells or cells treated with zVAD-fmk showed a few stained orange vesicles indicative of autophagy (images not shown for controls or cells treated with zVAD-fmk alone). Cells treated with Adriamycin alone showed a slight increase in the extent of orange vesicle staining; however, both MDA-MB231 cells and MCF-7/E6 cells treated with ZVAD-fmk + Adriamycin demonstrated extensive orange vesicular staining throughout the cytoplasm. Fig. 5C shows that essentially identical results were obtained upon staining with Monodansylcadaverine, another dye indicative of the formation of autophagosomes. These findings are similar to our previous reports where autophagy was induced in MCF-7 cells by a vitamin D_3 analog combined with ionizing radiation [33] or a novel microtubule poison [38]. We did not detect mitotic catastrophe (binucleated cells with micronuclei), another potential mode of cell death (data not shown).

There is an ongoing controversy in the scientific literature as to whether autophagy is cytoprotective or cytotoxic [6,7]. We therefore examined sensitivity to Adriamycin in the MDA-MB231 and MCF-7/E6 cells that had been treated with ZVAD-fmk and in which autophagy was detected. However, clonogenic survival assays indicated that sensitivity to Adriamycin was not altered when cells were treated with ZVAD-fmk (Table 1), indicating that in the case of Adriamycin treatment of cells lacking functional p53, autophagy is likely to be a mode of cytotoxicity when the apoptosis pathways is attenuated or abrogated.

3.5. *c-myc does not appear to mediate the senescence response to Adriamycin*

Fig. 2 indicates that c-myc protein levels were reduced in the p53 wild type MCF-7 cells as well as in the p53 mutant MDA-MB231 cells and p53 null MCF-7/E6 cells in association with senescence induced by Adriamycin. This observation is consistent with our earlier studies of Adriamycin effects on expression of c-myc and its correspondence with drug-induced growth arrest in MCF-7 cells [42]. As both p53 and $p21^{waf1/cip1}$ are known to suppress c-myc [27,28], and as c-myc inactivation has been reported to promote tumor regression through senescence [24], we evaluated the possibility that regulation of c-myc is involved in the accelerated senescence response to Adriamycin. Studies were performed in MCF-7 (35im) cells with inducible c-myc [36]. Fig. 6A demonstrates that c-myc levels were maintained by exposure of the cells to doxycyline alone as well as in the presence of Adriamycin treatment (although we did observe a transient unexplained transient decline at day 3). Fig. 6B indicates that Adriamycin promoted accelerated senescence in the MCF-7/35im cells despite the fact that c-myc levels were not suppressed. A reduction in senescence (of approximately 30%) was observed only at day 5.

Table 1 – Clonogenic survival assay following acute ADR exposure (% control).

	ADR	ZVAD + ADR
MCF-7/E6	2.9 ± 1.1	$2.7 \pm 1.1^{*}$
MDA-MB231	2.7 ± 0.9	$1.5 \pm 0.3^{**}$

Values represent mean number of colonies ± S.D. 2 weeks post-ADR exposure.
* $P = 0.8377$, MCF-7/E6 ADR vs. Zvad-fmk + ADR.
** $P = 0.1915$, MDA-MB231, ADR vs. Zvad-fmk + ADR.

3.6. *Involvement of reactive oxygen in the senescence response to Adriamycin*

As indicated above, $p21^{waf1/cip1}$ induction is considered a hallmark as well as a mediator of both accelerated and replicative senescence. Studies assessing the involvement of $p21^{waf1/cip1}$ in replicative senescence have implicated mitochondrial reactive oxygen generation downstream of $p21^{waf1/cip1}$ [29]. Reactive species such as hydrogen peroxide have been shown to promote senescence, presumably through the induction of p53 and $p21^{waf1/cip1}$ [44]. Furthermore, a number of studies have implicated reactive oxygen directly in the cytotoxic actions of Adriamycin [45–48]; however, it is generally thought that topoisomerase II is the primary drug target and that reactive oxygen plays a minor role, if any, in the direct, primary actions of Adriamycin in the tumor cells [49–51]. To evaluate the potential involvement of reactive oxygen species in the accelerated senescence response to Adriamycin, we determined whether the free radical scavenging agents, N-acetyl cysteine and glutathione could interfere with drug-induced senescence. Fig. 7(lower panel) indicates that both N-acetyl cysteine and glutathione are capable of suppressing the senescence response to Adriamycin in all three breast tumor cell lines studied, MCF-7, MDA-MB231 and MCF-7/E6 cells. The upper portion of the figure shows representative images of the senescent cells with and without exposure to N-acetyl cysteine or glutathione.

In an effort to link the suppression of senescence by GSH and NAC to the role of senescence in sensitivity to Adriamycin, studies were performed to assess sensitivity to Adriamycin in cells exposed to NAC or GSH. Fig. 8 shows that NAC and GSH

independent experiments. (C) Representative figures demonstrating autophagy by monodansylcadaverine dye staining in MDA-MB231 and MCF-7/E6 cells. (D) Quantification of the extent of autophagy in MDA-MB231 and MCF-7/E6 cells. *$P < 0.05$.

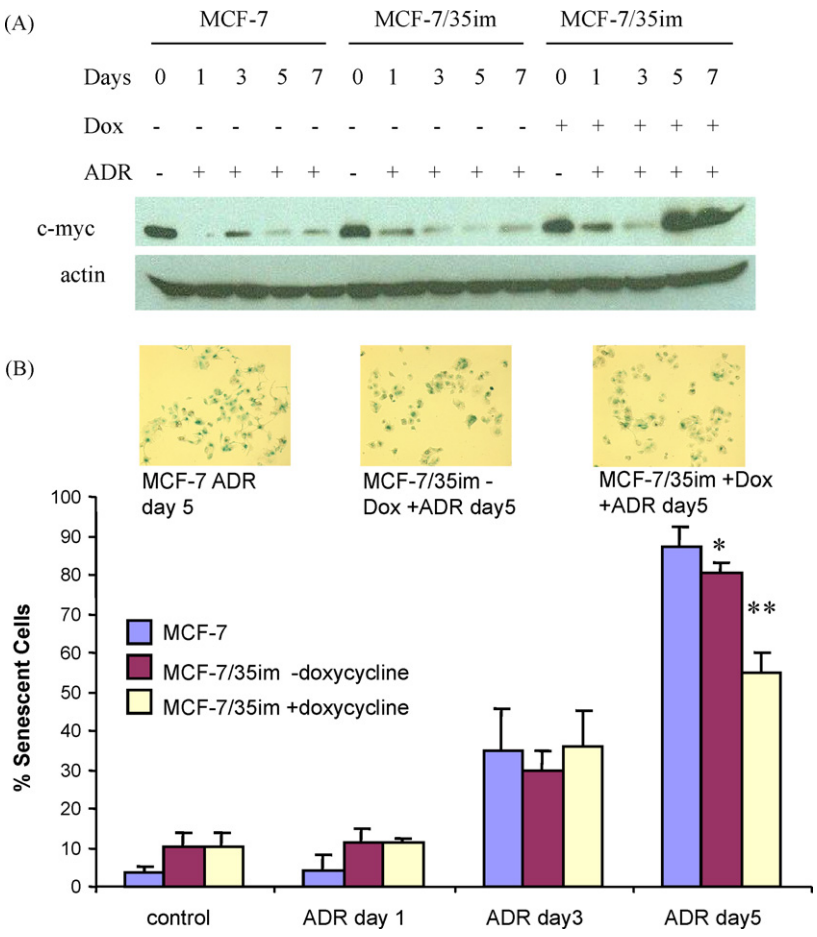

Fig. 6 – Senescence in c-myc inducible breast tumor cells. MCF-7/35im cells were either treated with 0.75 μM Adriamycin alone (2 h) or Adriamycin subsequent to induction with 1 μg/ml doxocycline. Upper portion of figure: c-myc levels in drug treated cells. Central portion: senescence in MCF-7 and MCF-7/35im cells treated with Adriamycin. Lower portion: quantitation of senescence. *P value of 0.2709, MCF-7 vs. MCF-7/35im – dox; **P value of 0.0026, MCF-7 vs. MCF-7/35im + dox.

protected the MCF-7 breast tumor cell line from Adriamycin action; interestingly, however, the apparent protection of MDA-MB231 and MCF-7/E6 cells did not rise to significance upon rigorous statistical analysis. It is noteworthy that the protection of MCF-7 cells was evident despite the fact that GSH and NAC were each alone slightly detrimental to cell viability.

To examine the possibility that NAC and or GSH might simply be binding the Adriamycin and thereby suppressing its interaction with the tumor cell, we performed a spectral analysis of Adriamycin when mixed with either NAC or GSH. However, the presence of either NAC or GSH failed to alter the intensity of the Adriamycin signal (data not shown).

4. Discussion

4.1. *Involvement of p53 and p21$^{waf1/cip1}$ in the accelerated senescence response to Adriamycin*

Our previous work strongly suggested that drug- and radiation-induced senescence in breast tumor cells is p53 dependent [20,52]. However, there is evidence that chemotherapeutic drugs can promote senescence even in cells lacking functional p53 [13,18,19]. Our current work indicate that while senescence is unlikely to be the preferred or predominant response to drugs such as Adriamycin in cells that are either mutant or null in p53, cells that survive the initial burst of cell death are capable of entering into a growth-arrested senescent state. In this context, Roberson et al. [13] reported that after the induction of senescence in p53 null lung cancer cells, a subpopulation was capable of proliferative recovery. Since we have also reported proliferative recovery in p53 wild type MCF-7 cells after promotion of senescence by Adriamycin [16], these findings may have implications for disease recurrence after drug and radiation treatment.

It is generally thought that whether or not p53 is involved, accelerated senescence is dependent on the induction of p21$^{waf1/cip1}$ [9]. Studies by Chang et al. [18] as well as our own unpublished work demonstrates attenuation of a senescence response to Adriamycin in HCT-116 cells where p21$^{waf1/cip1}$ has been silenced. Others have shown that induced expression of p21$^{waf1/cip1}$ promotes senescence [53] and that abrogation of p21$^{waf1/cip1}$ induction blocks senescence [43]. The induction of p21$^{waf1/cip1}$ that is observed in both the MDA-

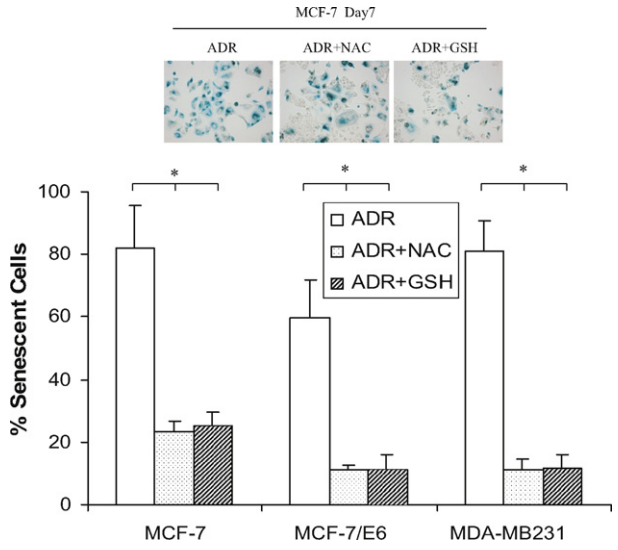

Fig. 7 – Influence of free radical scavengers on senescence induced by Adriamycin in MCF-7, MDA-MB231 and MCF-7/E6 breast tumor cells. MCF-7, MDA-MB231 and MCF-7/E6 cells were treated with either 10 mM glutathione or 10 mM N-acetyl cysteine for 1 h prior to exposure to 1 μM Adriamycin for 2 h and restoration of the NAC or GSH for the subsequent period of the experiment. Senescence was monitored based on cell morphology and expression of beta galactosidase. Upper portion of figure: beta galactosidase staining in MCF-7 cells. Lower portion of figure: quantitation of senescence in the absence and presence of GSH and NAC. *P < 0.05. Data was derived from three replicate experiments.

MB231 and the MCF-7/E6 cells clearly must be occurring in a p53-independent fashion, possibly through CHK2 [54–56]. Our studies using a luciferase reporter assay to detect evidence of p53 action through binding and activation of downstream promoters demonstrated that $p21^{waf1/cip1}$ induction is not due to the upregulation of functional wild type p53.

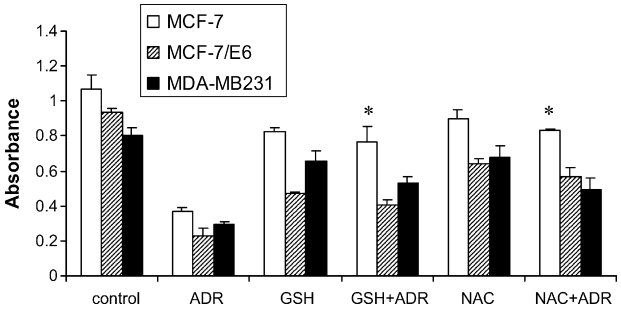

Fig. 8 – Protection of MCF-7 breast tumor cells from Adriamycin by NAC and GSH. MCF-7, MDA-MB231 and MCF-7/E6 cells were treated with 1 μM of Adriamycin in the presence or absence of 20 mM GSH or 20 mM NAC for 2 h, followed by removal of all drugs and washing of the cells. Three days post-Adriamycin treatment, the MTT assay was performed to assess the impact of GSH and NAC on tumor cell sensitivity to Adriamycin. Absorbance on the y-axis is an indication of cell number. *P < 0.05, ADR vs. GSH + ADR or ADR vs. NAC + ADR in MCF-7 cells.

4.2. *Suppression of cdc2 and c-myc in the accelerated senescence response to Adriamycin*

Work from our own as well as other laboratories [13,16,17,39,57] has suggested that accelerated senescence in response to chemotherapy could be associated with the down regulation of cdc2. Consistent with this previous work, we do detect suppression of cdc2 in MCF-7 cells as well as the MDA-MB231 and MCF-7/E6 cell lines. However, we remain cautious in the interpretation of these findings and the relationship of down regulation of cdc2 to the promotion of senescence in our experimental systems awaits further studies.

With regard to c-myc, our previous work demonstrated a strong association between suppression of the c-myc message and growth inhibition by Adriamycin [42] suggesting that c-myc might be linked to regulation of the senescence response. This concept is supported by a number of reports linking suppression of c-myc with the promotion of senescence [24–26]. However, sustained c-myc expression in the c-myc inducible MCF-7 cells failed to attenuate the senescence response to adriamycin, with the exception of day 5, when a reduction in the senescent population of approximately 30% was noted compared to the uninduced cells. In previous work, we reported that the accelerated senescence response to ionizing radiation was also not attenuated with c-myc induction [33]. Furthermore, HCT-116 cells demonstrate a robust and extensive senescence response to Adriamycin without detectable alterations in c-myc protein levels (unpublished data). Taken together, these findings suggest that the suppression of c-myc is likely to be only a minor component of the accelerated senescence to Adriamycin.

4.3. *Reciprocal regulation of alternative modes of cell death*

A number of studies have indicated that tumor cells can respond to stresses such as DNA damage through alternate and complementary pathways that suppress proliferative capacity and/or promote cell death. Schmitt et al. reported that a block to apoptosis in a murine lymphoma model resulted in a senescence response to cyclophosphamide [2]. Rebbaa et al. found that when p53 wild type SKN-SH human neuroblastoma cells were treated with doxorubicin and a caspase inhibitor, the cells switched from apoptosis to senescence, but cell killing was unaltered [34]. Park et al. reported that p53 mutant cells in which apoptosis induced by low dose Adriamycin was blocked were unable to maintain a senescence response, resulting in mitotic catastrophe [58]. Similarly, both Bunz et al. [31] and Lock et al. [30] reported that inhibition of apoptosis failed to diminish the cytotoxic effects of drug treatment due to alternative modes of cell death. Finally, knockout of either p53 or $p21^{waf1/cip1}$ function in colon carcinoma cells, perturbations that would change the ratio of senescent to apoptotic cells, failed to influence sensitivity to Adriamycin [59].

Our findings are generally quite consistent with these earlier studies in that a pharmacological blockade to apoptosis in p53 mutant MDA-MB231 cells or p53 null MCF-7/E6 cells

failed to reduce sensitivity to Adriamycin. Despite the fact that breast tumor cells lacking functional p53 are apparently competent to senesce [13,18], the blockade to apoptosis in either MCF-7/E6 cells or MDA-MB231 cells only moderately increased the extent of the senescence response in one of the two cell lines. Instead, the cells preferentially underwent autophagy. These findings indicate that when apoptosis is blocked and the cell seeks alternative modes of cell death, senescence is unlikely to be the preferred response, at least in the absence of functional p53. The fact that sensitivity to Adriamycin was unaltered strongly supports the likelihood that autophagy is killing the tumor cells rather than offering a means of cytoprotection under these experimental conditions. It is noteworthy that promotion of autophagy appears to occur both in p53 wild type cells [33] and in cells lacking functional p53, as in our previous study using a microtubule poison [38].

4.4. *Reactive oxygen generation in the action of Adriamycin and the promotion of senescence*

The role of reactive oxygen in the action of Adriamycin remains controversial. Although free radical-mediated toxicity is well established in the heart [47], the literature appears to overwhelmingly support a mechanism of action in tumor cells that is distinct from the generation of reactive oxygen species and that is associated primarily if not exclusively with poisoning of topoisomerase II [49,51]. With a few exceptions [46], studies supporting direct free radical generation by Adriamycin have been performed using inordinately high drug concentrations or somewhat unphysiological conditions, such as with serum free medium [48]. A study in xenografts of MDA-MB231 cells failed to detect evidence for iron-mediated production of reactive oxygen species by Adriamycin [50].

This said, it is both unexpected and intriguing that our studies found that both N-acetyl cysteine and glutathione markedly suppressed the senescence response to Adriamycin in both p53 wild type MCF-7 cells and the two cell lines lacking functional p53. The fact that NAC and GSH also protected the MCF-7 cells against Adriamycin toxicity supports the contention that accelerated senescence induced by Adriamycin plays a substantive role in the drug's antitumor effects. However, we cannot entirely rule out the possibility that these agents provide protection against additional modes of drug action. While it has been reported that N-acetyl cysteine attenuates the effects of doxorubicin on p53 induction, binding and other downstream targets [45], suggesting actions of this compound that are perhaps not directly related to suppression of free radical generation, this interpretation would not apply to the effects of the NAC in the breast tumor cells that are either mutant or null in p53. Since we detected only very low levels of reactive oxygen species in MCF-7 cells exposed to Adriamycin using DCF-DA staining (data not shown), it is possible (although still speculative) that the reactive oxygen that is putatively being suppressed is being generated outside the cell and acting in an autocrine fashion to promote senescence.

The fact that protection by NAC and GSH in the MDA-MB231 and MCF-7/E6 cells failed to achieve statistical significance is consistent with the fact that senescence plays a relatively limited role in the action of Adriamycin in these cells lacking functional p53, where apoptosis appears to be the primary response [20]. These studies furthermore support the premise while that reactive oxygen is involved in the promotion of accelerated senescence by Adriamycin, this is likely to be a secondary downstream response to drug treatment rather than a primary product of tumor cell exposure to Adriamycin.

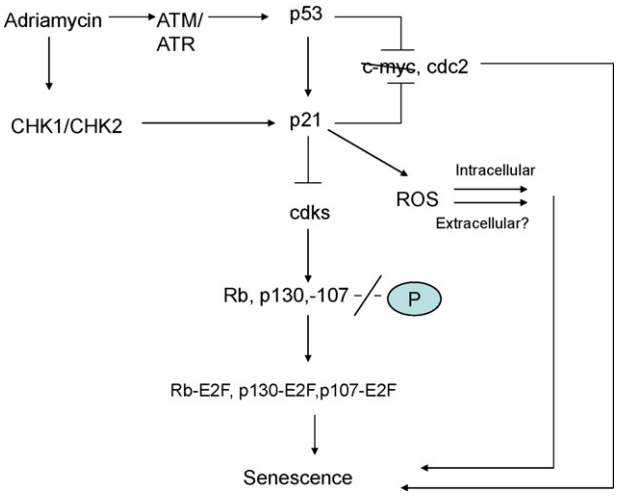

Fig. 9 – Overview. Diagrammatic representation of the signaling pathways that are putatively involved in the accelerated senescence response to Adriamycin.

Fig. 9 provides a diagrammatic representation of our findings and conclusions. Our studies are consistent with the involvement of p21 $^{waf1/cip1}$, cdc2 and reactive oxygen in the accelerated senescence response to Adriamycin in breast tumor cells. However, the relationship of these signaling elements and the source of the reactive oxygen remain to be defined. The dephosphorylation (and activation) of Rb and its family members p130 and p107 through inhibition of cyclin dependent kinases by upregulation of p21$^{waf1/cip1}$ leads to the formation of complexes with E2F family members that blocks cell cycle progression [15]. If c-myc plays a role in accelerated senescence induced by Adriamycin, its involvement is likely to be relatively modest. Apoptosis, senescence and autophagy all appear to have the capacity to suppress the reproductive capacity of the breast tumor cell, reflecting the multiple and complementary cell death pathways that the cell engages in response to DNA damage.

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