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A novel group of genes regulate susceptibility to anti-neoplastic drugs in highly tumorigenic breast cancer cells

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Running title: Genes regulating doxorubicin susceptibility in breast cancer

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Nonstandard abbreviations used in the paper:

Atf3- activating transcription factor 3

CAII- carbonic anhydrase II

Id2- inhibitor of differentiation/DNA binding 2

p55PIK- phosphatidylinositol 3-kinase 55 kDa regulatory sub-unit

RNAi- RNA inhibition

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Abstract

Doxorubicin is an anthracycline antibiotic used for cancer chemotherapy. The utility of doxorubicin is limited by its inability to kill all of the cells within a tumor and by resistant cells emerging from the treated population. We have screened for genes that regulate doxorubicin susceptibility in highly tumorigenic breast cancer cells by cDNA microarray and RNAi analysis, and have identified genes associated with both proliferation and cell cycle arrest following doxorubicin treatment. We confirmed that MDA-MB-231 cells treated with doxorubicin induce the expression of CAII (carbonic anhydrase II), Id2 (inhibitor of differentiation 2), Atf3 (activating transcription factor 3), and p55PIK (a phosphotidylinositol 3-kinase regulatory sub-unit). These genes were induced at different times and with varying specificities to different chemotherapeutic drugs. In addition to being induced at the transcriptional level, the CAII and clusterin proteins were elevated after doxorubicin treatment. CAII, Id2, p55PIK, and clusterin were not altered by doxorubicin in MCF-7 cells, a weakly tumorigenic cell line used in previous studies of doxorubicin-regulated gene expression. By inhibiting gene expression using RNAi, we found that CAII and clusterin increase cell survival following doxorubicin treatment, while Id2 increases susceptibility to doxorubicin. Our results support a model in which highly tumorigenic breast cancer cells induce a transcriptional response to doxorubicin that is distinct from less malignant cells. The induced genes regulate drug susceptibility positively and negatively and may be novel targets for therapeutic intervention.

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Solid tumors are typically treated with a regimen that includes DNA replication inhibitors (Chabner et al., 2001). Because tumors replicate at a high rate, the resulting DNA damage reduces the tumor mass and suppresses spread of the tumor. However, DNA replication inhibitors generally fail to kill all of the cells within a tumor, and the surviving cells frequently develop drug resistance. One of the primary goals in cancer research is to develop new ways of inhibiting cancer cell growth, in part by improving the effectiveness of existing cancer treatment regimens.

Doxorubicin (or adriamycin) is an anthracycline antibiotic that is a component of many treatment regimens for solid tumors. Doxorubicin blocks the activity of topoisomerase II, a DNA unwinding protein, causing arrest of the cell cycle or apoptosis (Chabner et al., 2001). Doxorubicin resistance can emerge through altered availability of the drug or through its inactivation, through changes in topoisomerase II, or through changes in pathways mediating DNA repair and apoptosis (Longley and Johnston, 2005). In principle, identification of genes that induced by doxorubicin could lead to new targets for improving the effectiveness of doxorubicin-based therapies.

Doxorubicin is a mainstay in the treatment of breast cancer, and several groups have used microarrays to screen for doxorubicin-regulated genes in breast cancer cell lines. Kudoh, *et al.* identified 14 genes that are up-regulated and 3 genes that are down-regulated by doxorubicin in MCF-7 breast cancer cells (Kudoh et al., 2000). MCF-7 cells are a p53/estrogen receptor (ER)/progesterone receptor (PR)-positive cell line that is non-tumorigenic in the absence of estradiol (Soule and McGrath, 1980). In general, genes that were up-regulated by doxorubicin, including cyclin D2 and Cdk6, direct cell cycle progression, while genes that were down-regulated, such as Bcl-2, inhibit apoptosis. The study by Kudoh, *et al.*, was performed with a

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microarray filter containing 5180 genes, and the authors anticipated further analyses when sequencing of the human genome was complete (Kudoh et al., 2000).

More recently, Troester, *et al*. analyzed global gene expression patterns in two different p53-positive breast cancer cell lines (one was the same MCF-7 cell line) treated with doxorubicin or 5-fluorouracil. These expression patterns were compared with those of mammary epithelial cells that were immortalized with telomerase (Troester et al., 2004). For MCF-7 cells, the results of Troester, *et al*. differed from those of Kudoh, *et al*. in that genes associated with proliferation, such as *CDC2*, cyclin A2, Ki67, and ribonucleotide reductase, were repressed, while cell cycle inhibitors like $p21^{WAF1}$ were induced. Similar results were found more recently by Elmore, et al. (Elmore et al., 2005). All three studies analyzed expression patterns in MCF-7 cells treated with similar doxorubicin doses (1 μM and 1.7 μM, respectively) and similar time points, and two studies detected the induction of epoxide hydrolase by doxorubicin (Kudoh et al., 2000; Troester et al., 2004).

Other groups have used microarray-based screens to search for doxorubicin-regulated genes in hepatoma cells (Moriyama et al., 2003), lung cancer cells (Niiya et al., 2003), lymphoblasts (Hussain et al., 2004), and in breast cancer cells treated with hepatocyte growth factor/scatter factor (Yuan et al., 2001). Because these studies spanned a range of cell types and conditions, doxorubicin-regulated genes varied widely. Additional studies have used microarrays to identify expression patterns associated with doxorubicin-resistant cells. These studies identified midkine (Kang et al., 2004) and eukaryotic translation initiation factor 1A (Kang et al., 2004), among others (Ichikawa et al., 2004; Kudoh et al., 2000), as important in acquired doxorubicin resistance. Finally, other groups have compared doxorubicin-treated cells that display a senescent morphology with cells that have continued to proliferate. Chang, *et al*.

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compared arrested and proliferating cells 10 days after a 24 hour dose of doxorubicin and identified numerous genes affecting proliferation and arrest (Chang et al., 2002). The regulation of many of these genes was p53-dependent (Chang et al., 2002).

Unlike previous studies in MCF-7 cells, we have determined a doxorubicin-specific expression pattern in the highly tumorigenic MDA-MB-231 cell line (Zhang et al., 1991), which is p53/ER/PR-negative. In MDA-MB-231 cells, doxorubicin induced genes that regulate numerous pathways, including cellular proliferation and survival, deacidification, and membrane signaling. Using our microarray data set as candidate genes, we then inhibited three of the genes using RNAi and tested their roles in anti-neoplastic drug susceptibility. Our results support a model in which tumorigenic breast cancer cells undergo a doxorubicin-induced change in gene expression that is distinct from less malignant cells, and we have identified multiple genes that regulate anti-neoplastic drug susceptibility.

MATERIALS AND METHODS

Cell culture and drug treatments. The HeLa, MCF-7, and MDA-MB-231 cancer cell lines were obtained from the American Type Culture Collection (ATCC) and maintained according to their instructions. Cells were maintained in 5% CO_2 at 37^{o}C. Doxorubicin, camptothecin, and etoposide were purchased from Sigma. Mechlorethamine was the kind gift from the laboratory of Dr. Robert Orlowski at the University of North Carolina at Chapel Hill.

For drug treatments, cells were split to a density of 500,000 cells per 100 mm dish and allowed to attach overnight. Cells were then treated with various drug concentrations and incubated for 24 hours. Cells were harvested by scraping from the dish with a rubber policeman, centrifuging, and washing once with phosphate-buffered saline. Doses of various drugs were

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chosen because they cause toxicity following a prolonged incubation (data not shown). However, after 24 hours, none of these agents caused pronounced cellular rounding, and the FACS profiles indicated that the cells were viable and largely non-apoptotic (Figure 1).

Microarray conditions. Three untreated or three doxorubicin-treated plates of cells were harvested separately, and RNA was purified from each treatment plate using the RNAeasy kit from Qiagen. Each purified RNA was then separately reverse transcribed, labeled, and hybridized to a Hu 133A chip (Affymetrix) by the University of Kentucky Microarray Core Facility. For each probe set, a two independent sample *t*-test was performed to test for the equality of the mean expression levels between the untreated and doxorubicin-treated cells. The assumption of equal variance between the two treatment groups was made. Of the 22,125 probe sets, 1502 probe sets had significant differences between the mean expression levels of the untreated and doxorubicin-treated cells with a significance of 0.01. Of the 1502 probe sets, 903 had a false discovery rate less than 0.01.

RT-PCR. RNA was purified as described above and reverse transcribed using SuperScipt II Reverse Transcriptase and random hexamers (both from Invitrogen). PCR reactions were performed with Taq polymerase (Genscript) in an Eppendorf Master Cycler using 30-38 cycles of a program consisting of 94^{o}C for 1 min., 55^{o}C for 1 min., and 72^{o}C for 1 min. PCR reactions contained primers to doxorubicin-induced genes and to actin, which was an internal control for the amount of cDNA template. DNA was then visualized by electrophoresis in 2.5% agarose 1000 (Invitrogen). The primer sequences for the various analyses are available on the web (www2.mc.uky.edu/Pharmacology/rjc_research.asp). Images of the agarose gels were captured as jpg files, and the intensities of the bands were quantitated using Image Quant

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software (Molecular Dynamics). The ratios of doxorubicin signature genes to actin were calculated, and statistical tests were performed using Microsoft Excel.

Cell cycle analysis. MDA-MB-231 cells were treated with various drugs, harvested, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol overnight. After fixing, the cells were washed again with PBS and resuspended at a density of 10^6 cells/ml in 20 μg/ml propidium iodide and 20 μg/ml DNase-free RNase. Samples were analyzed at the University of Kentucky Flow Cytometry Facility. The percentages of cells in the G_1, S, and G_2 phases of the cell cycle were calculated using ModFit software (Verity).

Western blotting. Cell lysates were prepared by incubating cells in NP-40 buffer (1% NP-40, 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, pH 7.4, and 10 μg/ml of the protease inhibitors aprotinin and leupeptin) followed by centrifugation at maximum speed in a microcentrifuge. Lysates were then separated on 8-20% SDS-PAGE gels. Proteins were then transferred to Immobilon P membranes (Millipore) and probed with various antibodies before visualization with the West Pico chemiluminescent substrate (Pierce). The antibodies to clusterin (H-330) and carbonic anhydrase II (H-70) were from Santa Cruz, and the antibody to tubulin was from Fisher.

RNAi transfections. MDA-MB-231 cells were plated at a density of 500,000 cells per 100 mm dish and left to attach overnight. After attachment, RNA oligonucleotide duplexes were diluted to 220 nM in 1 ml of Opti-MEM medium (Invitrogen) and left for 5 minutes. A 1:6 suspension of Oligofectamine (Invitrogen) in Opti-MEM was then added to the RNAi duplex solution, and the mixture was incubated at room temperature for 20 minutes. During the incubation, plated MDA-MB-231 cells were washed once with Opti-MEM and overlaid with 4.4 ml of Opti-MEM. The RNAi duplex suspension was gently added to the cells, for a final

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concentration of 40 nM RNAi duplex. Following a 4 hour incubation at 37^{o}C, 2.8 ml of culture medium containing 30% Serum Supreme (Fisher) was added to the cells, and the cells were left overnight. The cells were then trypsinized from the dish and were plated at a density of 5000 cells per well in a 96-well dish or 500,000 cells per plate in a 100 mm dish.

Viability assays. For measurements of cell growth, transfected cells were counted manually and plated in triplicate in 96 well dishes, then treated with varying doses of doxorubicin, camptothecin, etoposide, or mechlorethamine for 96 hours. Media were then removed and replaced with growth media containing 0.5 mg/ml MTT (3-[4,5 dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide, Sigma) and incubated for 1-2 hours. Following incubation, MTT-containing media were removed and 100 µl of dimethyl sulfoxide was added to each well. The cells were then incubated for 20 minutes on a rotating platform and the A_{595}/A_{650} was determined using a Dynatech MR600 Microplate Reader. The percent viability was calculated as absorbance of cells treated with drug divided by the average absorbance of untreated cells. In each case, the results shown indicate representative results of at least three independent experiments. LD_{50} values were calculated from triplicate MTT assay measurements.

RESULTS

The doxorubicin transcriptome in MDA-MB-231 breast cancer cells includes 903 genes. To examine doxorubicin-mediated gene expression in a highly tumorigenic cell line, we treated MDA-MB-231 human breast cancer cells with 1 µM doxorubicin for 24 hours. At this time and dose, the cells were not apoptotic but were arrested in the S and G_2/M phases of the cell cycle (Figure 1, panels B and F). The resulting microarray analysis identified 4266 genes out of

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22,283 that were significantly altered by doxorubicin (P<0.01). A subset of 903 genes were selected as the only genes with a false discovery rate of <1% (see Methods).

The doxorubicin-regulated genes were comprised of multiple classes of genes, some of which are listed in Table I. When sorted by annotation of biological function, there was a significant up- and down-regulation of metabolic genes and genes regulating cell cycle progression (See Supplementary data). Genes regulating DNA synthesis and chromatin assembly were significantly induced (Fisher exact test, P=0.03, respectively), while repressed genes included regulators of chemotaxis (P=0.0005), ion transport (P=0.03), and the inflammatory response (P=0.04; see Supplementary data). Because of the global patterns of transcriptional changes, we have analyzed the expression of two transcription factors previously associated with proliferation (Id2 and Atf3) and two metabolic genes regulating acid-base metabolism (CAII) and phospholipid synthesis (p55PIK). We note that the microarray predicted that the most strongly up-regulated gene was KRML (Kreisler Maf-related leucine zipper homologue, Table 1), but we were unable to replicate this result in independent PCR analyses (data not shown).

Doxorubicin-regulated genes are induced with varying kinetics and drug specificities. To examine the kinetics of gene induction, we analyzed RNA levels by reverse transcription-polymerase chain reaction (RT-PCR) using actin as an internal standard for cDNA loading. CAII and Id2 were induced quickly by doxorubicin, with a detectable increase 2-6 hours after treatment (Figure 2A and 2B). In contrast, p55PIK and Atf3 were induced more slowly, reaching peak expression only after 24 hours (Figure 2C and 2D, respectively). These results suggest that doxorubicin-regulated genes are induced at different rates.

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Doxorubicin causes arrest of the cell cycle in the S and G_2/M phases (Figure 1B). We determined whether induction of doxorubicin signature genes is cell cycle phase-specific. Like doxorubicin, the topoisomerase II inhibitor etoposide (Chabner et al., 2001) caused arrest in S and G_2/M (Figure 1, panels B, D, and F, respectively), while the topoisomerase I inhibitor camptothecin (Chabner et al., 2001) arrested cells in G_1 and G_2/M (Figure 1, panels C and F). The alkylating agent mechlorethamine, the active form of cyclophosphamide, which requires activation in the liver (Chabner et al., 2001), arrested cells primarily in G_1 and S (Figure 1, panels E and F).

Expression was analyzed following low or high doses of drugs (Figure 3A-D), and then the same assays were performed in triplicate for statistical analysis (Figure 3E-H). Carbonic anhydrase II (CAII) was induced efficiently by multiple drugs (Figure 3A). The induction of CAII by doxorubicin, camptothecin, etoposide, and mechlorethamine was significant when compared to untreated cells ($P \leq 0.01$ for each drug), but not when CAII induction by any two drugs (i.e. doxorubicin and camptothecin) was compared ($P > 0.05$ for any pair). Id2 induction by doxorubicin was highly significant (Figure 3B, lanes 1-3; $P = 2 \times 10^{-6}$ for untreated vs. treated cells, Figure 3F), and Id2 was induced to a lesser degree by camptothecin (Figure 3B, lanes 4-5; 5-fold), etoposide (Figure 3B, lanes 6-7; 8-fold), and mechlorethamine (Figure 3B, lanes 8-9; 10-fold). In each case, the levels of induction by doxorubicin were significantly higher than for camptothecin, etoposide, and mechlorethamine ($P < 0.0001$ for each, Figure 3F).

We observed similar results for p55PIK and Atf3, where doxorubicin specifically induced p55PIK (Figure 3C and G) and Atf3 (Figure 3D and H). In these cases, relatively low levels of induction were detected in cells treated with camptothecin, etoposide, and mechlorethamine. The results indicate that doxorubicin induces some genes with a remarkable degree of specificity,

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while other genes are induced by DNA damaging agents that do not share the cell cycle arrest profile of doxorubicin.

The CAII and clusterin proteins are induced by doxorubicin. As a consequence of its transcriptional regulation by doxorubicin, the CAII protein was induced by 1 μM doxorubicin (Figure 4A) and by camptothecin (Figure 4C, lanes 2 and 3). CAII levels following treatment with etoposide or mechlorethamine were lower than levels following doxorubicin and camptothecin treatment (Figure 4C, lanes 4 and 5), which is consistent with the RNA analysis for CAII (Figure 3). Clusterin regulates chemotherapy resistance in osteosarcoma (Trougakos et al., 2004), and we found that clusterin was highly induced by 0.1 μM doxorubicin and was nearly saturated by a 0.5 μM dose (Figure 4B). In addition, clusterin was highly induced by all four chemotherapeutic agents that we tested (Figure 4D). We did not analyze Id2, Atf3, or p55PIK by western blot because commercially available antibodies were not sufficiently specific.

Most of the doxorubicin-regulated genes in MDA-MB-231 cells are not altered by doxorubicin in MCF-7 cells. Previous microarray analyses of doxorubicin-treated breast cancer cells utilized MCF-7 cells and did not detect the majority of the genes identified in our screen. To examine differences in gene regulation in MDA-MB-231 and MCF-7 cells directly, we treated both cell lines with doxorubicin and analyzed differences in gene expression. In some cases, we also analyzed changes in HeLa cells, which have been widely used in studying drug resistance. MCF-7 cells expressed high levels of CAII (Figure 5A, lane 1), and CAII levels did not change with doxorubicin treatment (Figure 5A, lane 2). In contrast, MDA-MB-231 and HeLa cells expressed low levels CAII (Figure 5A, lanes 3 and 5) and induced CAII expression after doxorubicin treatment (Figure 5A, lanes 4 and 6). CAII expression was so much higher in MCF-7 cells that the western blot had to be dissected and developed separately. In contrast,

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clusterin was not expressed in MCF-7 cells before or after doxorubicin treatment (Figure 5B, lanes 1 and 2) but was efficiently induced by doxorubicin in both MDA-MB-231 and HeLa cells (Fig. 5B, lanes 3-6).

Like CAII, Id2 was expressed highly in MCF-7 cells (Fig. 5C, lane 3), and its expression did not increase after doxorubicin treatment (Fig. 5C, lane 4). Similar to clusterin, p55PIK was expressed at low levels in MCF-7 cells (Fig. 5D, lane 3), and p55PIK expression decreased in MCF-7 cells after doxorubicin treatment (Fig. 5D, lane 4). The only gene with a similar regulation in MDA-MB-231 and MCF-7 cells was Atf3, which was minimally expressed in both cell lines (Fig. 5E, lanes 1 and 3) and was induced by doxorubicin (Fig. 5E, lanes 2 and 4).

CAII, Id2, Atf3, and clusterin regulate drug susceptibility. To determine the roles of doxorubicin-induced genes, we attenuated the expression of several genes using RNAi oligonucleotide duplexes (Elbashir et al., 2001). CAII, Id2, and clusterin expression was inhibited by transiently transfecting MDA-MB-231 cells with RNAi duplexes targeting the genes (CAIIi, Id2i, or CLSi) or with a non-specific control sequence (con, Fig. 6). RNAi molecules targeting CAII, Id2, and clusterin inhibited the basal expression of Id2 (Fig. 6B, compare lanes 1 and 3) or attenuated the induction of each gene after doxorubicin treatment (Fig. 6A-C, compare lanes 2 and 4). Furthermore, CAII protein levels were markedly decreased before (Figure 6D, lanes 1 and 2) and after doxorubicin treatment (Figure 6D, lanes 3 and 4) in CAIIi-transfected cells.

Cells with inhibited clusterin expression exhibited a loss of viability compared to control-transfected cells following doxorubicin treatment (Figure 7A). The change in viability in CLSi- vs. control-transfected cells at 0.08-2 μM doxorubicin was significant ($P \leq 0.007$ for each dose). There was no change in viability in CLSi-transfected cells without drug treatment, consistent

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with the low levels of basal clusterin expression in this cell line (Figure 4B, lane 1; Figure 5B, lane 3; Figure 6C, lane 1). The loss of viability in CLSi-transfected cells resulted in a significant decrease in LD_{50} compared to control cells following treatment with doxorubicin, camptothecin, etoposide, and mechlorethamine (Figure 7C; $P \leq 0.01$ for each). We conclude that clusterin suppresses susceptibility to multiple chemotherapeutic drugs in MDA-MB-231 cells.

Inhibition of CAII expression increased susceptibility of MDA-MB-231 cells to doxorubicin, camptothecin, etoposide (Figure 7B, solid line), and mechlorethamine. The differences between control- and CAIIi-transfected cells were highly significant at doses of 1.25 μM (P=0.04), 5 μM (P=0.0001), and 20 μM etoposide (P=0.002). Similar to clusterin, the LD_{50} values for cells with inhibited CAII expression were significantly lower than control cells following treatment with doxorubicin, camptothecin, etoposide, and mechlorethamine (Figure 7C, $P \leq 0.005$ for each). It is likely that this is a conservative estimate of the effect of CAII on drug susceptibility, because there was a low level of CAII expression in the CAIIi-transfected cells (Figure 6D, lane 4). We conclude that CAII expression is associated with decreased susceptibility to multiple chemotherapeutic drugs.

Id2 inhibition decreased cell proliferation (Fig. 8A, dark columns), even in the absence of doxorubicin. Growth inhibition was highly significant at 25 nM ($P=2 \times 10^{-7}$) or 100 nM ($P=8 \times 10^{-5}$) doses and was reflected in a 46% decrease in the viable cell count 96 at hours post-transfection. Id2 inhibition did not induce a significant increase in cell death, because we detected only a modest increase in the number of dead cells (5 ± 2 for control-transfected cells compared to 15 ± 5 for Id2i-transfected cells) by trypan blue exclusion assay. In contrast, the Id2i-transfected population contained a 12% increase in the number of cells arrested in S, G_2, and M phase (data not shown) when measured by FACS analysis. Id2i-transfected cells

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exhibited decreased sensitivity to doxorubicin (Figure 8B). At the 0.3 μM dose of doxorubicin, Id2i-transfected cells were significantly less sensitive than cells transfected with the control RNAi duplex (Figure 8B, P=0.002). As a result, Id2i-transfected cells had LD_{50} values greater than control cells for doxorubicin (Figure 7C), but not for camptothecin, etoposide, or mechlorethamine. We conclude that Id2 expression is associated with doxorubicin susceptibility in MDA-MB-231 cells.

DISCUSSION

We have found that highly tumorigenic MDA-MB-231 breast cancer cells treated with chemotherapeutic drugs initiate a transcriptional response that is distinct from that of MCF-7 cells. Even in the presence of extensive damage and delayed cell cycle progression, cells induce the expression of genes associated with proliferation and survival. Genes induced by doxorubicin positively or negatively regulate drug susceptibility and also regulate cell proliferation. We will discuss these genes in the following sections.

Carbonic anhydrase II regulates the cellular acid/base balance by catalyzing the reaction:

$$H^+ + HCO_3^- \leftrightarrow H_2O + CO_2$$

This reaction allows for efficient secretion of acid (Potter and Harris, 2003). Tumors have an acidic extracellular pH, which may contribute to drug resistance by reducing the uptake and cytotoxicity of weak bases like doxorubicin (Raghunand et al., 1999). Our results are consistent with a model in which MDA-MB-231 cells induce CAII to improve survival, perhaps by limiting doxorubicin uptake. However, we note that CAII contributed to survival following treatment with camptothecin, etoposide, and mechlorethamine, suggesting a more universal mechanism for CAII. For instance, CAII may influence water or ion exchange with the extracellular

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environment. Numerous genes regulating ion and water balance were altered following doxorubicin treatment, including Aqp3/aquaporin and multiple solute carrier proteins (Table 1), suggesting that changes in membrane permeability and ion balance contribute to chemotherapeutic responsiveness. Finally, CAII was expressed more strongly in MCF-7 cells than in MDA-MB-231 cells and was not induced by doxorubicin in the former cell line (Figure 5A), suggesting that distinct pathways regulate CAII transcription in the two cell lines.

Several genes with reported anti-apoptotic functions were induced by doxorubicin in MDA-MB-231 cells. Clusterin/ Apolipoprotein J/ complement lysis inhibitor is a secreted protein that is induced by chemotherapy (Biroccio et al., 2003). Clusterin is overexpressed in tumors (Chen et al., 2003; Redondo et al., 2000), and has anti-apoptotic functions in some cell types (July et al., 2004; Trougakos and Gonos, 2002). Although we did not detect a net effect on proliferation following clusterin inhibition (data not shown), clusterin inhibition had a pronounced effect on drug susceptibility in MDA-MB-231 cells (Figure 7). Furthermore, clusterin was neither expressed constitutively nor induced in MCF-7 cells, suggesting that strategies targeting clusterin may be effective in highly tumorigenic cells. For all RNAi studies, we emphasize that changes in viability are conservative estimates, because transient transfections inhibit gene expression in less than 100% of the transfected cells.

Id2 (*i*nhibition of differentiation/inhibitor of *D*NA binding) is a basic helix-loop-helix (bHLH) protein that lacks a DNA binding domain and is capable of forming inactive heterodimers with other bHLH proteins (Sikder et al., 2003). Id2 is associated with proliferation, is up-regulated in cancers, and extends keritinocyte lifespan (Sikder et al., 2003). Id2 induction by doxorubicin was reported in earlier studies of murine fibroblasts, where it interfered with activation of the MyoD muscle-specific transcription factor (Kurabayashi et al., 1994). In

developing breast tissue, Id2 has been associated with proliferation (Mori et al., 2000) and differentiation (Miyoshi et al., 2002; Parrinello et al., 2001), but in human breast tumor samples, Id2 is frequently down-regulated (Itahana et al., 2003), and Id2 expression is associated with positive prognosis in breast cancer (Stighall et al., 2005). To clarify the role of Id2 in breast cell proliferation, Itahana, *et al*. expressed Id2 in MDA-MB-231 cells under a constitutive promoter, and Id2 suppressed cell cycle progression and invasiveness (Itahana et al., 2003). Our results using RNAi for Id2 are inconsistent with these overexpression studies, because Id2 inhibition suppressed proliferation. In some cases, constitutive overexpression of a gene phenotypically resembles the loss of the same gene, suggesting a sensitive regulation of the gene product. Indeed, Id2 expression is transcriptionally regulated during the cell cycle (Barone et al., 1994), and Id2 is phosphorylated by multiple kinases (Hara et al., 1997; Nagata et al., 1995), suggesting that constitutive expression of Id2 during G_1 phase could delay cell cycle progression.

Id2 inhibition improved cell survival following doxorubicin, suggesting that MDA-MB-231 cells induce Id2 as part of a pathway that leads to cell death. The mechanism through which Id2 regulated cell survival may include displacing other proteins from the Rb complex, which has been linked to chemotherapy susceptibility. However, doxorubicin-treated cells accumulate in S and G2/M (Figure 1), a stage where Rb is hyperphosphorylated and has minimal protein interactions through its pocket domain. An alternate model is that Id2 up-regulates cell cycle progression through binding to helix-loop-helix proteins. As a result, loss of Id2 expression decreases proliferation and improves survival, perhaps by allowing cells with damaged DNA more time to complete DNA repair.

MCF-7 cells undergo a senescent-like change when treated with doxorubicin, while MDA-MB-231 cells do not (Elmore et al., 2002). A doxorubicin-resistant MCF-7 sub-clone

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overcame senescence by limiting the repression of positive cell cycle regulators (Elmore et al., 2005), including cdc2 and cyclin E2, which are normally suppressed by doxorubicin (Elmore et al., 2005). In contrast, Id2 (a gene associated with proliferation) caused decreased survival in MDA-MB-231 cells, the opposite result. We conclude that MCF-7 cells suppress chemotherapy susceptibility through a distinct mechanism from MDA-MB-231 cells.

Phosphatidylinositol 3-kinase (PI3K) has anti-apoptotic functions in response to numerous stimuli (Fresno Vara et al., 2004), and we detected marked up-regulation of the PI3K regulatory sub-unit p55PIK/PI3K-p55γ (Pons et al., 1995) by doxorubicin. While the role of p55PIK in growth regulation is poorly understood, p55PIK binds to the Rb tumor suppressor protein (Xia et al., 2003), and overexpression of the amino-terminal Rb binding sequence of p55PIK causes growth arrest (Hu et al., 2005). p55PIK is also implicated in insulin and cytokine signaling (Dey et al., 1998; Mothe et al., 1997; Takahashi-Tezuka et al., 1997). In addition, we note that p55PIK was not induced by doxorubicin in MCF-7 cells, suggesting genetic factors that are altered in MDA-MB-231 cells regulate p55PIK expression.

Several other genes that are associated with proliferation were induced by doxorubicin. Atf3 is a member of the ATF/CREB (activating transcription factor/cAMP responsive element binding protein) family of bZip transcription factors (Hai and Hartman, 2001). Atf3 binds to the DNA sequence TGACGTCA and is associated with neoplastic transformation and the response to serum, stress, and damage (Amundson et al., 1999; Hai and Hartman, 2001; Shtil et al., 1999; Yu et al., 1996). Atf3 was up-regulated by doxorubicin in MDA-MB-231 and MCF-7 cells, suggesting that strategies targeting Atf3 would be unlikely to be specific for aggressive cancers.

Other pro-survival genes that were induced by doxorubicin include TRAIL-R3 and TRAIL-R4, which are “decoy receptors” for TRAIL, the tumor necrosis factor-related apoptosis

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inducing ligand (Kim and Seol, 2003). TRAIL-R3 and TRAIL-R4 bind to TRAIL but lack cytoplasmic death domains and do not induce apoptosis when engaged by TRAIL (Sheridan et al., 1997). Instead, TRAIL-R3 and TRAIL-R4 have anti-apoptotic functions (Bernard et al., 2001; Meng et al., 2000). TRAIL-R3 has been reported previously as a doxorubicin-responsive gene in breast cancer (Ruiz de Almodovar et al., 2004), and our findings also implicate TRAIL-R4 in resistance to doxorubicin-mediated cell death. In addition, numerous genes that modulate cell death were down-regulated by doxorubicin, including multiple interleukins. Among these genes, IL-1α has been investigated previously for its ability to enhance the anti-cancer activity of doxorubicin (Monti et al., 1993; Nakamura et al., 1991).

Doxorubicin also induced the expression of proteins that metabolize xenobiotic compounds, including the cytochrome P450 proteins Cyp1A1 and Cyp1B1, the cytochrome P450 reductase, and the cytochrome b_5-related P450 activator Iza1/Hpr6.6 (Table 1). Cyp1A1 and Cyp1B1 are induced in response to numerous aromatic compounds with similar structures to doxorubicin (Nebert and Russell, 2002). Iza1 homologues activate and stabilize cytochrome P450 proteins (Mallory et al., 2005; Min et al., 2004), and Iza1 regulates cell death following oxidative damage (Hand and Craven, 2003), which is generated by doxorubicin. As a group, these proteins likely modify doxorubicin or cellular metabolites induced by doxorubicin. Cytochrome P450 proteins likely act in concert with the drug transporter proteins GCN20/ABCF2 and MDRTAP, which were induced by doxorubicin (Table 1), in minimizing the effective concentrations of doxorubicin within the cell.

In summary, we have identified a group of genes that are induced by chemotherapeutic agents and which regulate drug susceptibility. The functions of individual genes could not be predicted based on their transcriptional pattern, because genes that were induced by doxorubicin

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had both positive and negative effects on doxorubicin susceptibility. In spite of this limitation, we detected two genes, CAII and clusterin, that were associated with cell survival following chemotherapeutic drug treatment. Because these genes were not transcriptionally altered in less tumorigenic cells, there may be a "therapeutic window" in which their activity can be inhibited in tumor cells without disrupting their function in non-tumorigenic cells. A third gene, Id2, is associated with increased doxorubicin susceptibility, suggesting that Id2 might serve as positive prognostic indicator when it is induced by doxorubicin in clinical tumor samples.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Cell cycle arrest following treatment with four different chemotherapeutic drugs. MDA-MB-231 cells were either left untreated (A) or were treated with 1 μM doxorubicin (B), 1 μM camptothecin (C), 10 μM etoposide (D), or 40 μM mechlorethamine (E) for 24 hours. The cells were then fixed and stained for DNA content with propidium iodide. The percentages of cells in G_1, S, and G_2/M were then calculated using ModFit software and are presented in (F). Doxorubicin and etoposide are both inhibitors of topoisomerase II and arrested the cell cycle in S

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and G_2/M (B, D, and F). Camptothecin is a topoisomerase I inhibitor that caused G_1 and G_2/M arrest, and mechlorethamine is an alkylating agent that arrested the cells in G_1 and S phase.

Fig. 2. Analysis of doxorubicin-induced genes by reverse transcriptase-polymerase chain reaction (RT-PCR). MDA-MB-231 breast cancer cells were treated with 1 μM doxorubicin and harvested at 0, 2, 6, 16, 24, and 48 hours after treatment (lanes 1-6). At each time point, RNA was purified and reverse transcribed. cDNA was then amplified with primers to actin (bottom bands) as a loading control and with primers to carbonic anhydrase II (CAII, A), Id2 (B), p55PIK (C), or Atf3 (D). The earliest time of induction varied among the four genes, with Id2 and CAII being stably induced within six hours, while p55PIK and Atf3 were induced strongly after 24 hours.

Fig. 3. Doxorubicin-induced genes differ in drug specificity. MDA-MB-231 cells were either left untreated (lanes 1), or were treated with 0.2 μM doxorubicin (lanes 2), 1 μM doxorubicin (lanes 3), 0.2 μM camptothecin (lanes 4), 1 μM camptothecin (lanes 5), 2 μM etoposide (lanes 6), 10 μM etoposide (lanes 7), 10 μM mechlorethamine (lanes 8), or 40 μM mechlorethamine (lanes 9). RNA was then purified and reverse transcribed. cDNA was then amplified with primers to actin (bottom bands) and with primers to carbonic anhydrase II (CAII, A), Id2 (B), p55PIK (C), or Atf3 (D). In E-H, RT-PCR reactions for doxorubicin-induced genes were performed in triplicate, then were quantitated, and the ratios of gene signal to actin were calculated. The genes analyzed were CAII (E), Id2 (F), p55PIK (G), and Atf3 (H). Cells were untreated (first columns, Un) or treated with 1 μM doxorubicin (second columns, Dx), 1 μM camptothecin (third columns, Cp), 10 μM etoposide (fourth columns, Et), or 40 μM mechlorethamine (fifth columns, MC).

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Each of the four genes was induced most highly by doxorubicin, and the ratio of each gene to actin in doxorubicin-treated cells is expressed as 100%. Error bars represent the standard deviation in three independent assays. The results showed that CAII is induced by multiple drugs, while Id2, Atf3, and p55PIK were induced primarily by doxorubicin.

Fig. 4. Western blot analysis of proteins encoded by doxorubicin-induced genes. For A and B, MDA-MB-231 cells were treated with 0, 0.1, 0.5, or 1 μM doxorubicin (lanes 1-4, respectively) for 24 hours, then were lysed and analyzed by western blot for CAII (A), clusterin (B), or tubulin (bottom panel) as a control for loading. The numbers to the left of each panel refer to the migration of stained molecular weight standards in kDa. Doxorubicin increased the expression of both proteins. For C and D, MDA-MB-231 breast cancer cells were left untreated (lanes 1) or treated with 1 μM doxorubicin (lanes 2), 1 μM camptothecin (lanes 3), 10 μM etoposide (lanes 4), or 40 μM mechlorethamine (lanes 5). The blot was probed for CAII (C), clusterin (D), or tubulin (lower panel).

Fig. 5. Four doxorubicin-induced genes are not altered in MCF-7 cells. A and B, proteins were analyzed by western blot in MCF-7 (lanes 1-2), MDA-MB-231 (lanes 3-4), and HeLa cells (lanes 5-6) that were untreated (odd lanes) or treated with 1 μM doxorubicin for 24 hours (even lanes). The blot was probed for CAII (A), clusterin (B), or tubulin (lower panel). CAII expression was significantly higher in MCF-7 cells without doxorubicin treatment (A, lane 1), and CAII expression did not change following doxorubicin (A, lane 2). In contrast, CAII was weakly expressed in MDA-MB-231 and HeLa cells (A, lanes 3 and 5), but both cells induced CAII expression after doxorubicin treatment (A, lanes 4 and 6). Clusterin was similarly induced by

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doxorubicin in MDA-MB-231 and HeLa cells (B, lanes 3-6), but was neither expressed constitutively nor induced by doxorubicin in MCF-7 cells (B, lanes 1 and 2). For C-E, RNA levels in MDA-MB-231 (lanes 1 and 2) and MCF-7 (lanes 3 and 4) cells were analyzed by RT-PCR using actin as an internal standard, before and after treatment with 1 μM doxorubicin. (C) Id2 expression increased with doxorubicin treatment in MDA-MB-231 cells (lanes 1 and 2), but was elevated and did not change with treatment in MCF-7 cells (lanes 3 and 4). (D) p55PIK expression increased with doxorubicin exposure in MDA-MB-231 cells (lanes 1 and 2), but was weak in MCF-7 cells (lane 3) and decreased with doxorubicin (lane 4). (E) Atf3 was minimally expressed in MDA-MB-231 and MCF-7 cells under normal growth conditions (lanes 1 and 3) and increased in both cell lines upon doxorubicin treatment (lanes 2 and 4).

Fig. 6. Doxorubicin-induced genes are inhibited by RNAi. Gene expression was analyzed by RT-PCR, which showed diminished expression of CAII (A), Id2 (B), and clusterin (C). In each case, the inhibited gene is the upper band, and the expression of actin (lower band) served as a control for loading. In lanes 1 and 2, expression was analyzed in cells transfected with a control oligonucleotide duplex in the absence (lane 1) or presence (lane 2) of 1 μM doxorubicin for 24 hours. In lanes 3 and 4, expression was analyzed in cells transfected with gene-specific RNAi duplexes in the absence (lane 3) or presence (lane 4) of 1 μM doxorubicin. In panel D, the inhibition of CAII was confirmed by western blot for CAII (upper panel), with tubulin as a control for loading (lower panel). Expression was analyzed in cells transfected with a control oligonucleotide duplex (con, lanes 1 and 3) in the absence (lane 1) or presence (lane 3) of 1 μM doxorubicin for 24 hours. In lanes 2 and 4, expression was analyzed in cells transfected with

gene-specific RNAi duplexes in the absence (lane 2) or presence (lane 4) of 1 μM doxorubicin. The results show that the various RNAi duplexes inhibit doxorubicin-induced gene expression.

Fig. 7. Clusterin, CAII, and Id2 regulate viability following treatment with chemotherapeutic drugs. (A) Inhibition of clusterin increases susceptibility to chemotherapeutic drugs, including doxorubicin. Equal numbers of MDA-MB-231 cells were transfected with control (Con, dashed line) or clusterin-specific (CLSi, solid line) oligonucleotide duplexes, then treated with increasing doses of doxorubicin for 72 hours. Viability was measured by MTT assay (Methods). (B) Inhibition of CAII increases susceptibility to multiple drugs, including etoposide. Cells were transfected as for panel A, and viability was measured by MTT assay. In panels A and B, all points were analyzed in triplicate and were repeated in three separate experiments. (C) LD_{50} values for MDA-MB-231 cells treated with a control RNAi duplex (left column), CAIIi (second column), CLSi (third column), or Id2i (right column). Id2i decreased susceptibility to doxorubicin ($P=0.0005$), while CLSi and CAIIi increased susceptibility to all four drugs ($P \leq 0.01$) for each.

Fig. 8. Id2 positively regulates proliferation and doxorubicin susceptibility. (A) MDA-MB-231 cells were transfected with a control RNAi (light gray bars) or Id2i (dark bars) and viability was determined by MTT assay. Id2i inhibited growth at doses of 25 or 100 μM. (B) Equal numbers of MDA-MB-231 cells transfected with a control RNAi duplex or Id2i were treated with increasing doses of doxorubicin and incubated for 72 hours. Viability was then measured by MTT assay. The results show that cells with inhibited Id2 expression (solid line) are significantly less susceptible to doxorubicin ($P=0.003$ at 0.3 μM dose) than cells transfected with

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the control RNAi (dashed line). The results are representative of five separate experiments.

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Table 1. Genes regulated by doxorubicin in MDA-MB-231 cells

Gene name	Fold change	P value[a]
Transcription factors		
Fos-b	80	0.001
ID-2, inhibitor of DNA binding	64	0.0002
ATF3, activating transcription factor 3	20	2×10^{-9}
c-Fos	6	0.0001
c-Myb	6	0.0001
EHF, Ets homologous factor	-13	0.0002
Cell signaling		
p55PIK, phosphatidylinositol 3-kinase regulatory subunit	12	0.0006
Rac3, Rho family GTP binding protein	-13	0.0004
EGFR, epidermal growth factor receptor	-5	0.0004
DNA structure		
Histone H3	46	1×10^{-7}
Histone H2A	14	6×10^{-6}
DNA Repair		
GADD-45β	13	0.0002
GADD-45γ	8	0.0001
NBS1, nibrin	-8	3×10^{-5}
OGG1	-5	0.0001
RAD50	-4	0.0003

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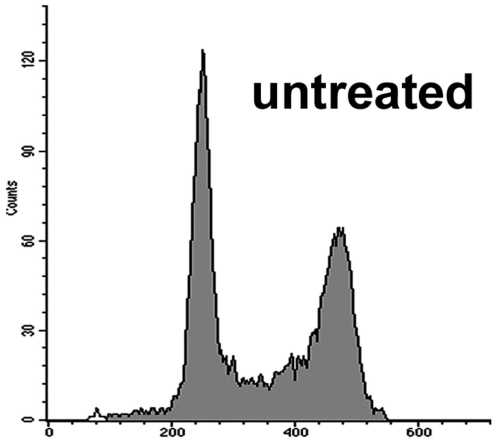
Cell death		
Clusterin; CLI, cytolysis inhibitor	64	0.0007
TRAIL-R3	8	0.0006
Death-preventing kinase	7	2×10^{-6}
TRAIL-R4	5	0.0004
Cell cycle regulation		
Cyclin-dependent kinase inhibitor 1, p21	10	0.0001
Cyclin-dependent kinase inhibitor 2, p18	9	0.0009
Cell division cycle 25C	4	0.0008
Cyclin A1	4	9×10^{-5}
Xenobiotic metabolism and drug transport		
ABCF2, ATP binding cassette (GCN20)	62	6×10^{-5}
MDRTAP, ATP binding cassette	8	6×10^{-6}
Epoxide hydrolase	8	0.008
Cyp1B1, cytochrome P450 protein 1B1	6	0.0004
Cyp1A1, cytochrome P450 protein 1A1	5	0.0001
NADPH-dependent cytochrome P450 reductase	5	3×10^{-6}
Iza1/Hpr6.6, cytochrome P450 activator	3	0.001
Deacidification/solute transport		
Carbonic anhydrase II	55	6×10^{-7}
AQP3, aquaporin 3	18	9×10^{-5}

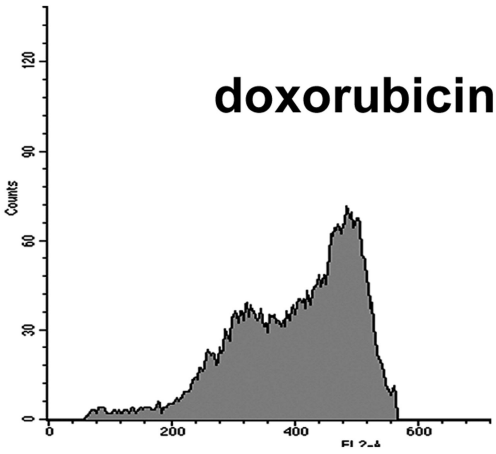
[a]Fold change indicates relative expression in doxorubicin-treated vs. untreated cells.

Figure 1

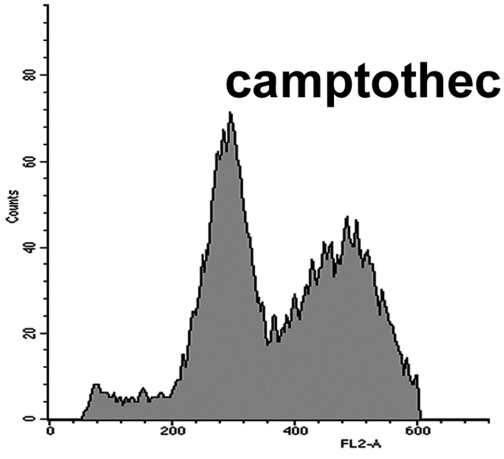
A.



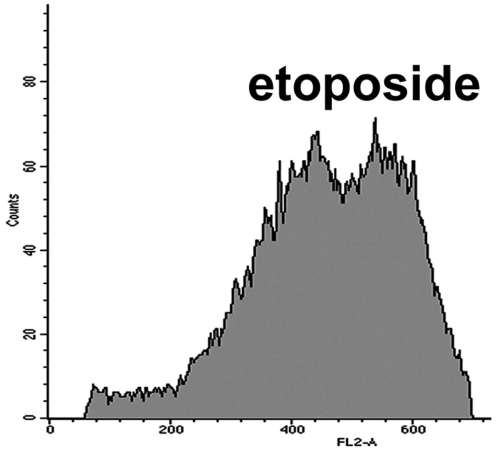
B.



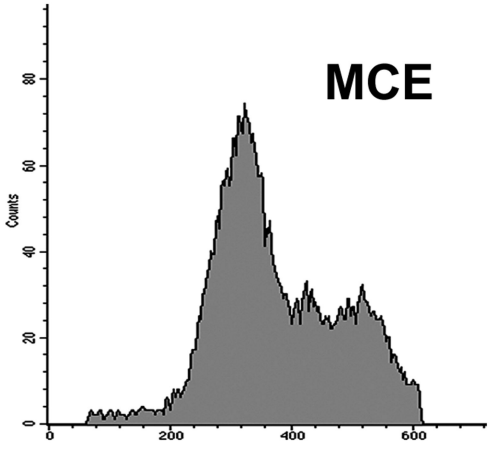
C.



D.



E.



F.

	G_1	S	G_2
No drug	38%	28%	34%
Doxorubicin	0.2	43	57
Camptothecin	40	5	55
Etoposide	0	53	47
Mechlorethamine	44	56	0

Figure 2

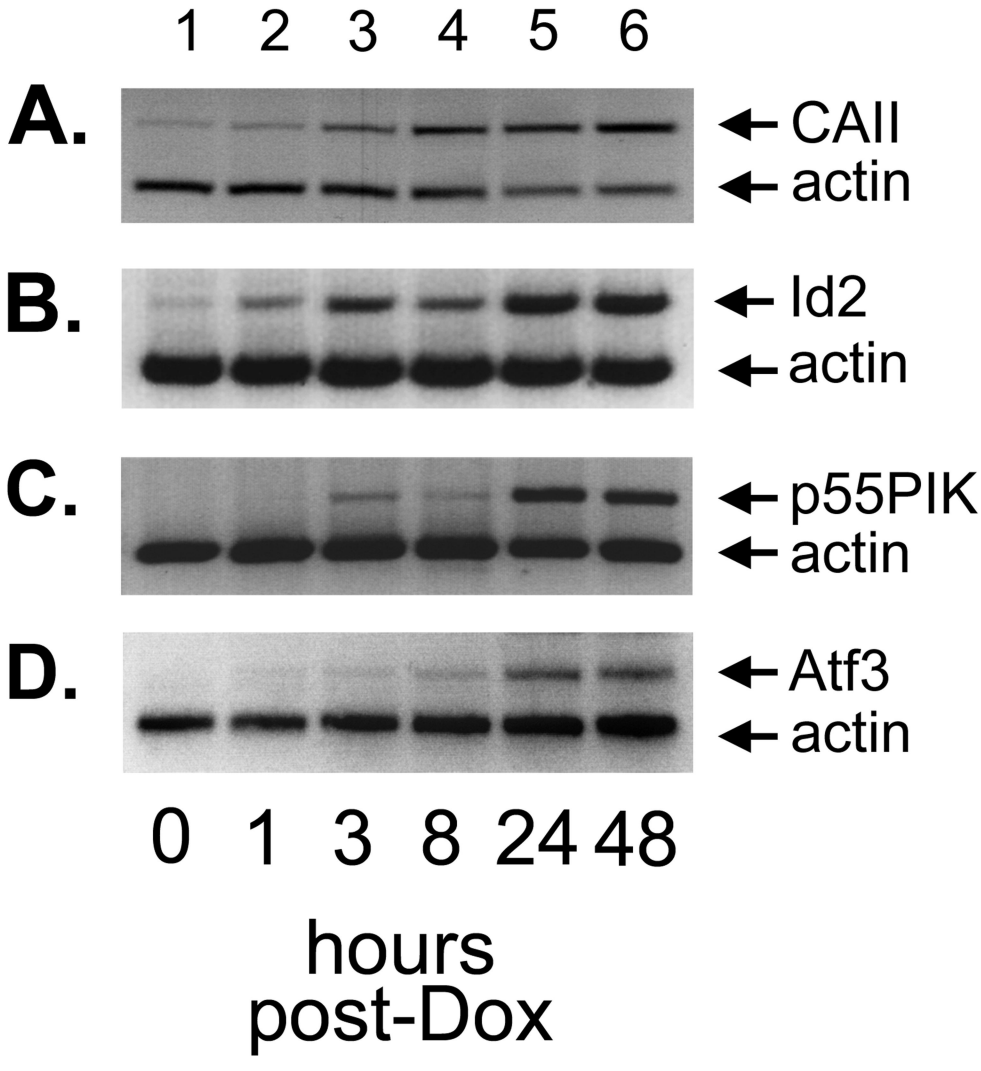
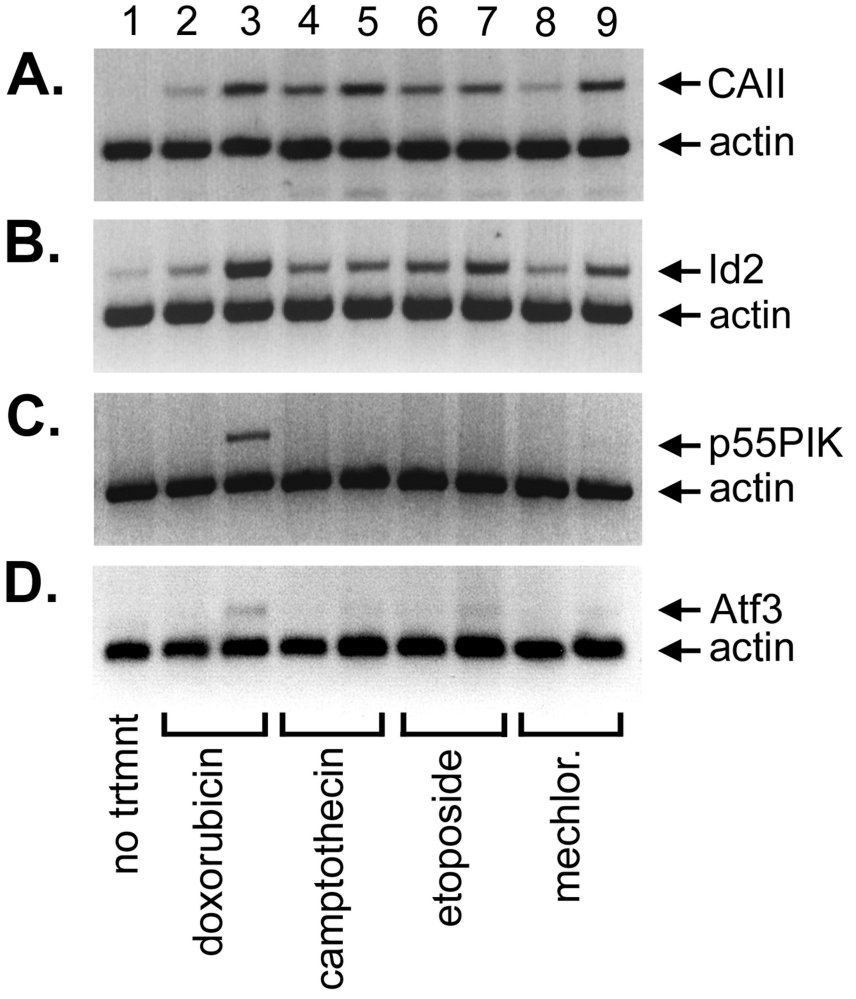

1 2 3 4 5 6
A.
CAII
actin
B.
Id2
actin
C.
p55PIK
actin
D.
Atf3
actin
0 1 3 8 24 48
hours
post-Dox

Figure 3



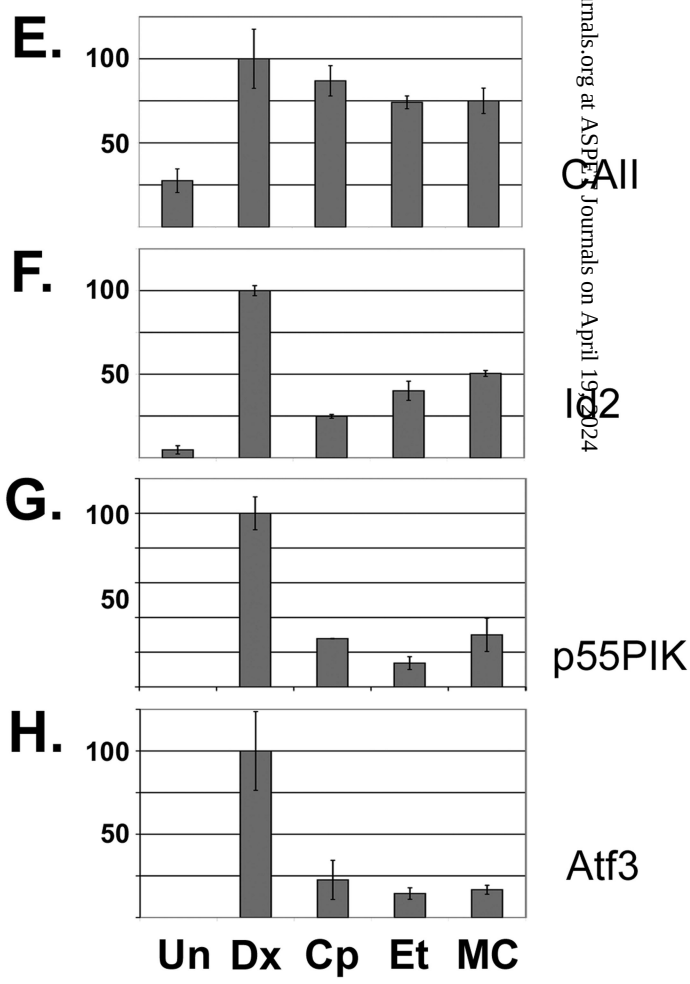


Figure 4

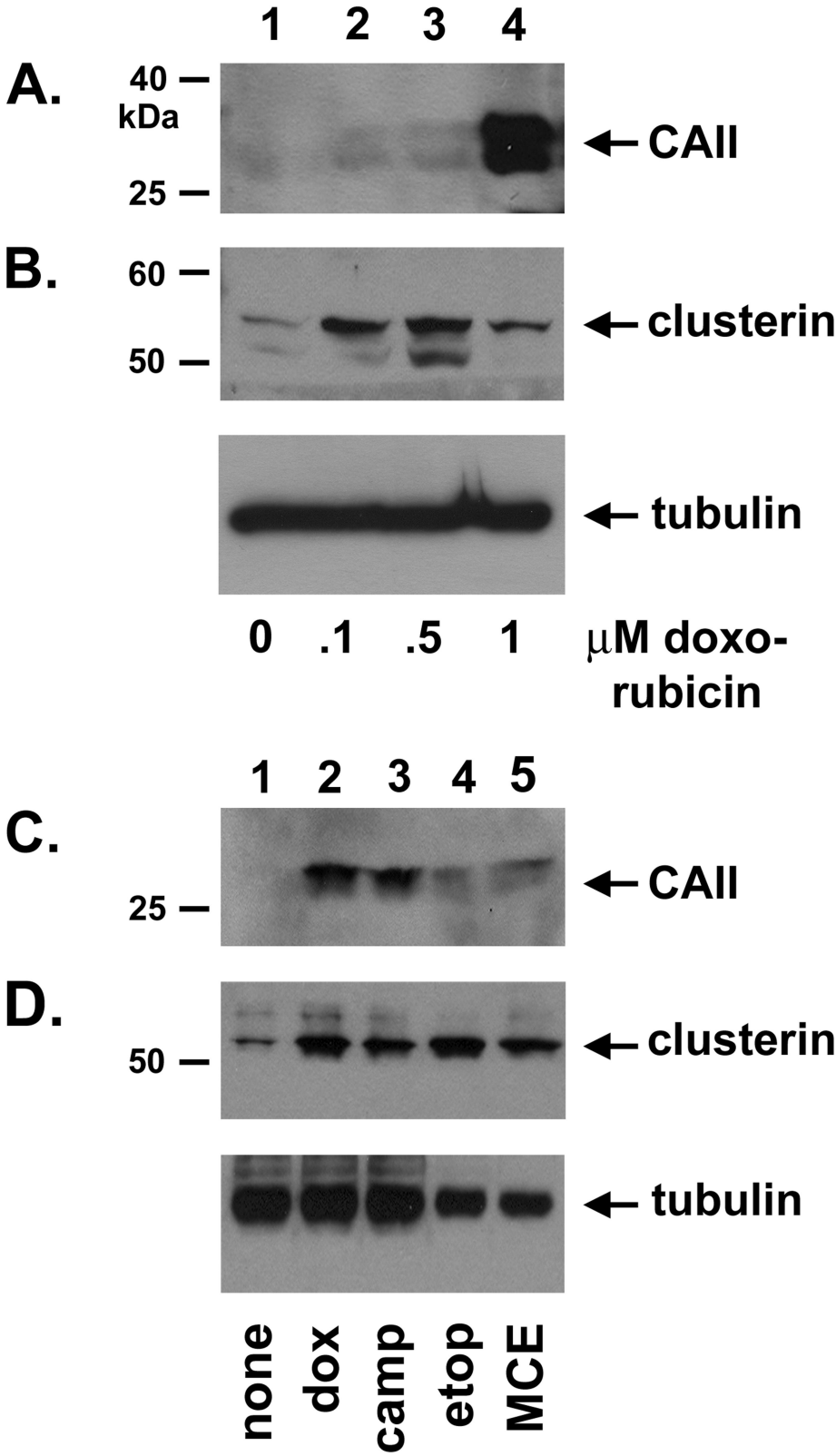

1 2 3 4
A.
40 kDa
25
CAII
B.
60
50
clusterin
tubulin
0 .1 .5 1
μM doxo-rubicin
1 2 3 4 5
C.
25
CAII
D.
50
clusterin
tubulin
none
dox
camp
etop
MCE

Figure 5

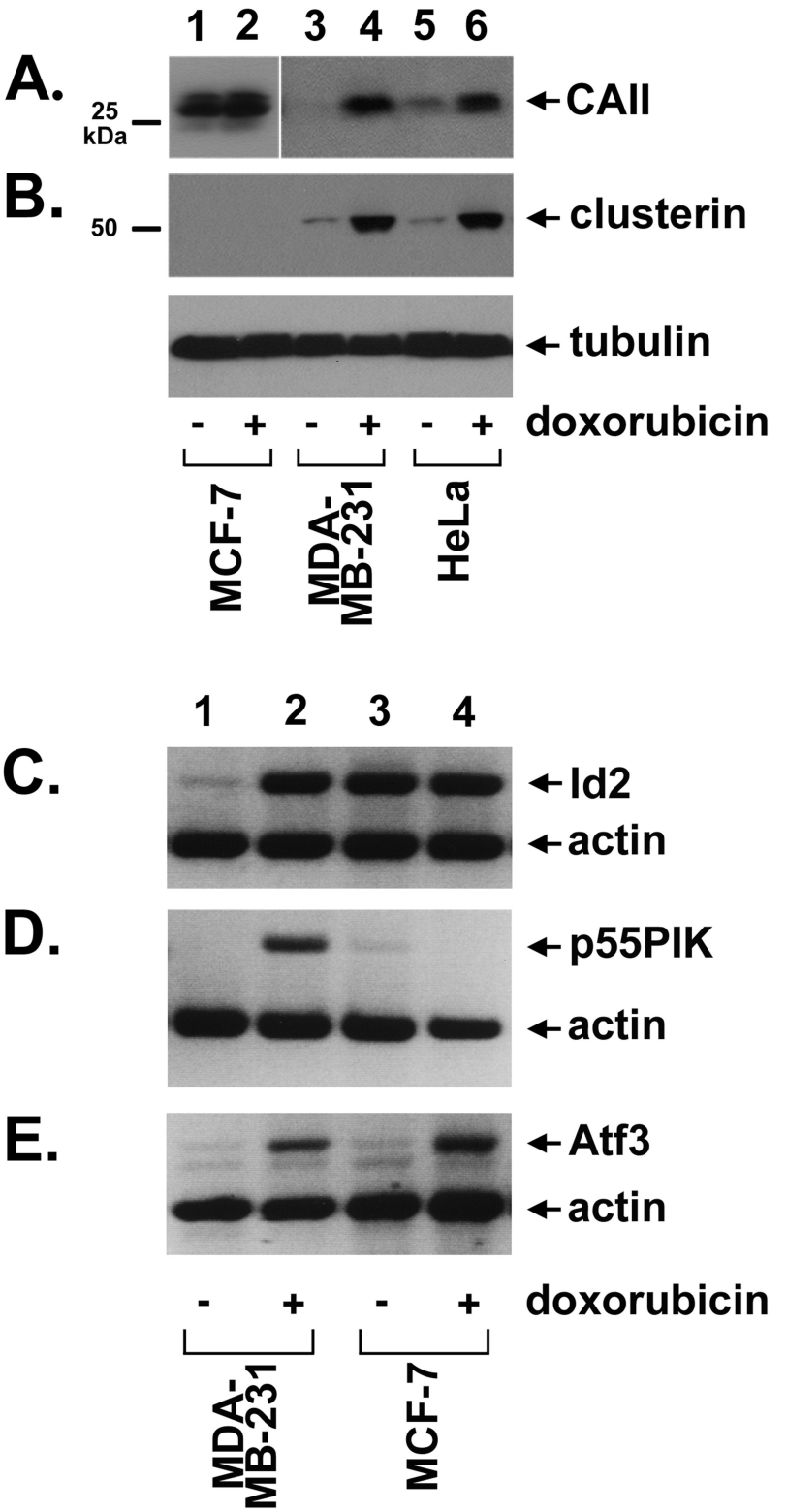
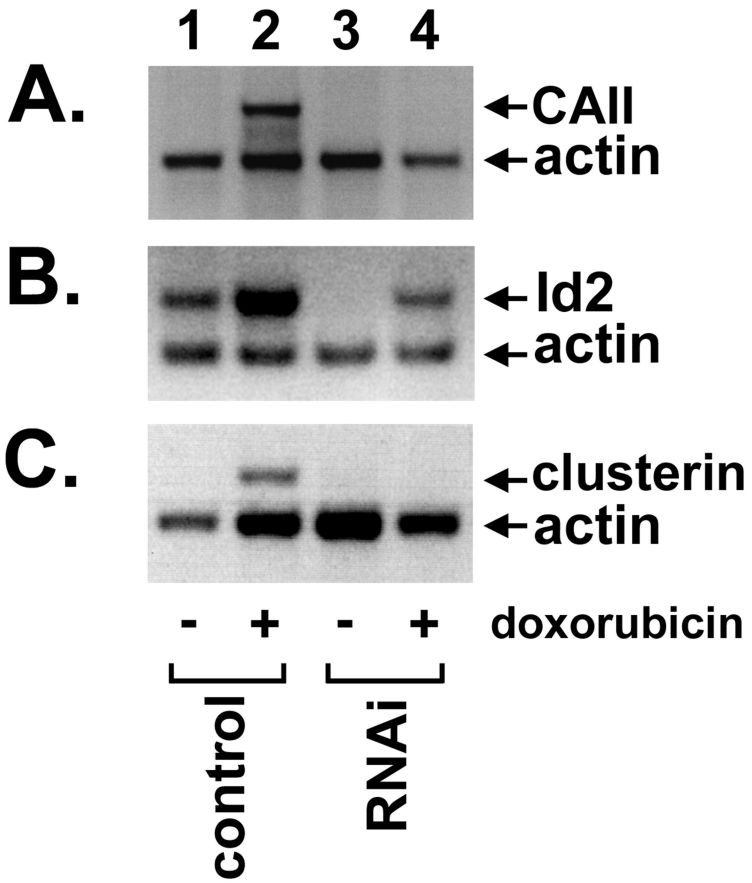

1 2 3 4 5 6
A.
25 kDa
CAII
B.
50
clusterin
tubulin
- + - + - +
doxorubicin
MCF-7
MDA-MB-231
HeLa
1 2 3 4
C.
Id2
actin
D.
p55PIK
actin
E.
Atf3
actin
- + - +
doxorubicin
MDA-MB-231
MCF-7

Figure 6



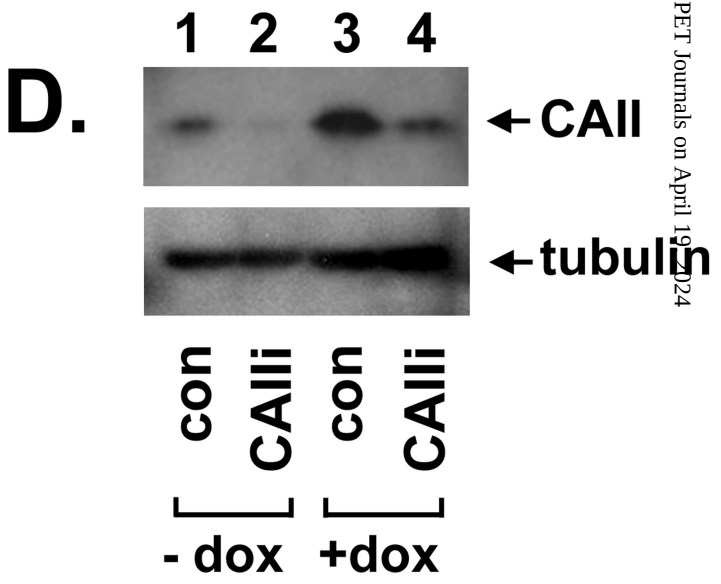
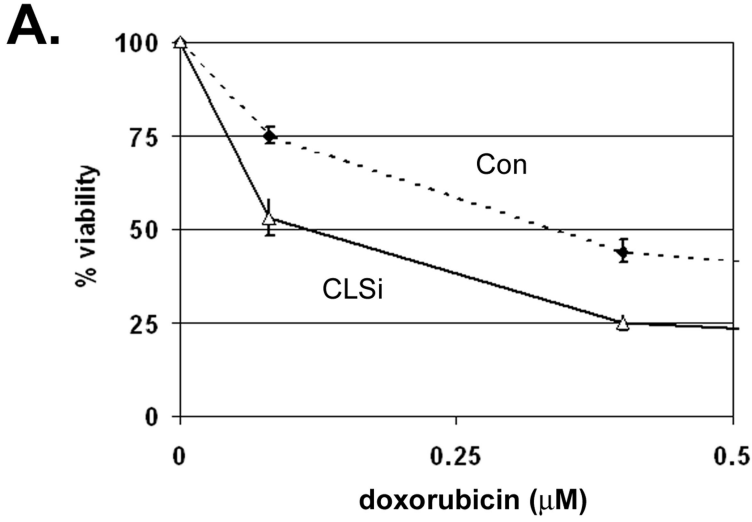
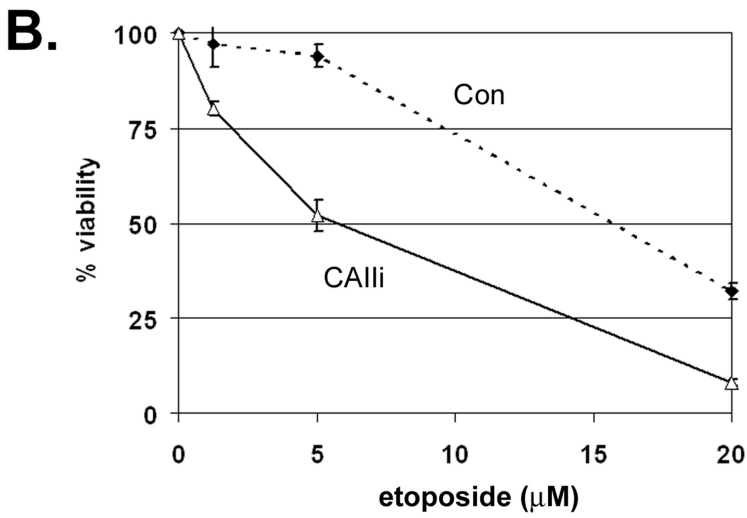


Figure 7


A.
100
75
50
25
0
% viability
Con
CLSi
0
0.25
0.5
doxorubicin (μM)


B.
100
75
50
25
0
% viability
Con
CAIIi
0
5
10
15
20
etoposide (μM)

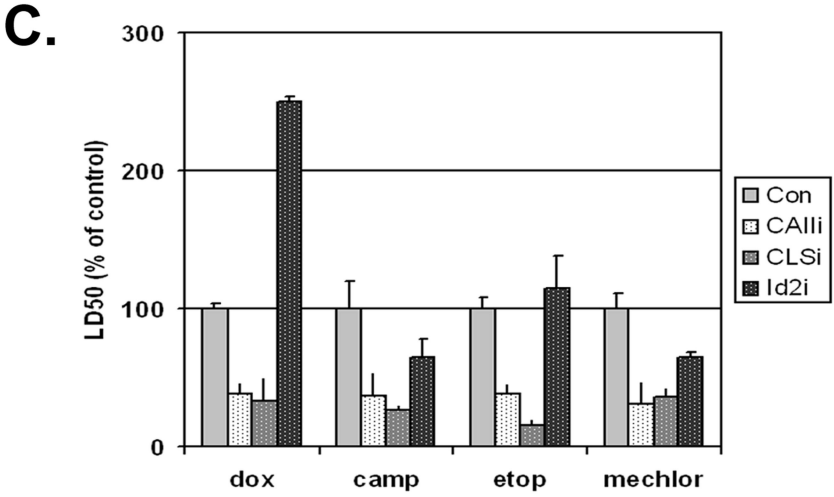
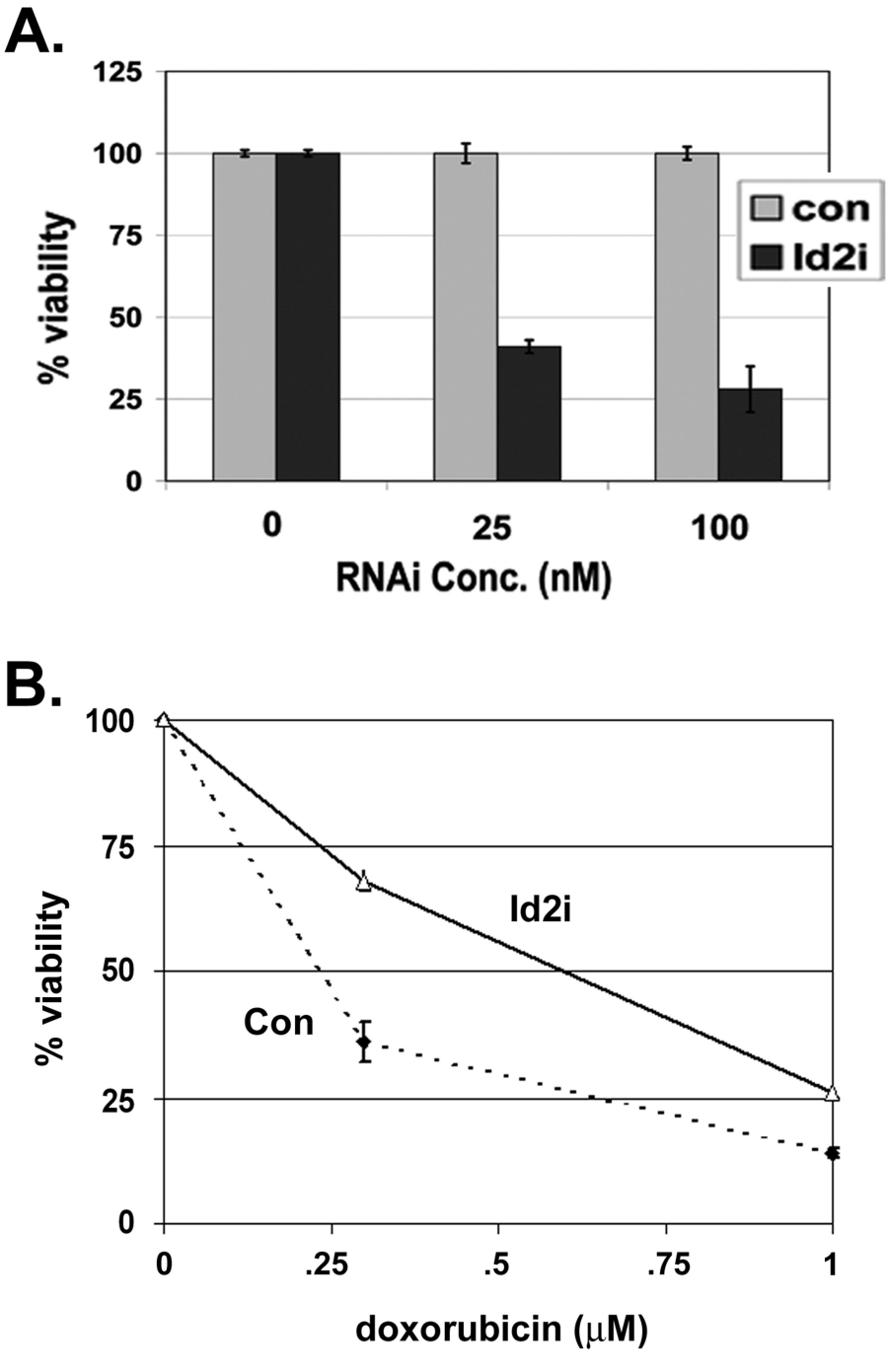

C.
300
200
100
0
LD50 (% of control)
Con
CAIIi
CLSi
Id2i
dox
camp
etop
mechlor

Figure 8


A.
125
100
75
50
25
0
% viability
con
Id2i
0
25
100
RNAi Conc. (nM)
B.
100
75
50
25
0
% viability
Id2i
Con
0
.25
.5
.75
1
doxorubicin (μM)