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A correlation between cytotoxicity and reductase-mediated metabolism in cell lines treated with doxorubicin and daunorubicin

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ABBREVIATIONS: AKR, aldo-keto reductase; CBR, carbonyl reductase; DOX, doxorubicin; DAUN, daunorubicin; DOXol, doxorubicinol; DAUNol, daunorubicinol.

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ABSTRACT

The role of metabolism in DAUN- and DOX-associated toxicity in cancer patients is dependent upon whether the parent drugs or major metabolites, doxorubicinol (DOXol) and daunorubicinol (DAUNol), are the more toxic species. Therefore, we examined whether an association exists between cytotoxicity and the metabolism of these drugs in cell lines from nine different tissues. Cytotoxicity studies using MTT cell viability assays revealed that four cell lines [HepG2 (liver), HCT-15 (colon), NCI-H460 (lung), and A-498 (kidney)] were more tolerant to DAUN and DOX than the five remaining cell lines [H9c2 (heart), PC-3 (prostate), OVCAR-4 (ovary), PANC-1 (pancreas), and MCF-7 (breast)], based on significantly higher LC_{50} values at incubation times of 6, 24, and 48 hrs. Each cell line was also assessed for its efficiency at metabolizing DAUN and DOX. The four drug-tolerant cell lines converted DAUN/DOX to DAUNol/DOXol more rapidly than the five drug-sensitive cell lines. We also determined whether exposure to DAUN or DOX induced an increase in metabolic activity among any of these nine different cell types. All nine cell types showed a significant increase in their ability to metabolize DAUN or DOX, in response to pre-exposure to the drug. Western blot analyses demonstrated the increased metabolic activity toward DAUN and DOX correlated with a greater abundance of eight aldo-keto and two carbonyl reductases following exposure to either drug. Overall, our findings indicate an inverse relationship between cytotoxicity and DAUN or DOX metabolism in these nine different cell lines.

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INTRODUCTION

The anthracyclines, daunorubicin (DAUN) {(8*S*,10*S*)-8-acetyl-10-[(2*S*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,8,11-trihydroxy-1-methoxy-5,7,8,9,10,12-hexahydrotetracene-5,12-dione} and doxorubicin (DOX) {(8*S*,10*S*)-10-[(2*R*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-5,7,8,9,10,12-hexahydrotetracene-5,12-dione}, are two of the most highly effective antineoplastic agents ever developed (Licata et al., 2000; Lakhman et al., 2005; Blanco et al., 2008), and they are widely used to treat a variety of cancers. However, their clinical application is severely limited because they are known to cause a number of adverse toxic side effects, the most serious of which is cardiotoxicity which can lead to congestive heart failure (CHF) (Wojtacki et al., 2000; Mordente et al., 2001; Danesi et al., 2002; Barry et al., 2007; Deng and Wojnowski, 2007; Menna et al., 2007). Cardiotoxicity appears to have two phases: an acute phase, in which symptoms develop during treatment, but are transient; and a latent phase, in which cardiac damage progresses over time, eventually presenting as permanently decreased left ventricular ejection fraction (LVEF) or even CHF (Brouwer et al., 2006). However, there is considerable variability in whether patents develop these cardiomyopathies; some patients are able to tolerate higher, more effective doses without manifesting any cardiac damage. Thus, if the basis of the interpatient sensitivity could be determined, the treatment could be tailored to provide the most effective dose for each patient.

The mechanism(s) that underlie both the antineoplastic and adverse effects of these anticancer drugs are unproven, but may include: (i) oxidative stress, including the formation of reactive oxygen species and/or cell membrane damage *via* lipid peroxidation; (ii) intercalation into nucleic acids causing suppression of DNA, RNA, and protein synthesis; (iii) induction of

DNA damage through interference with topoisomerase II; (iv) disturbances of calcium balance and iron homeostasis in myocardial cells; (v) induction of apoptosis; (vi) rise in cardiac histamine release; and (vii) generation of toxic metabolites (Klugmann et al., 1986; Gewirtz, 1999; Hrdina et al., 2000; Minotti et al., 2004; Simůnek et al., 2009; Mordente et al., 2009; Sawyer et al., 2010). Ample evidence has been published both supporting and countering each of these proposed mechanisms of anthracycline-induced cellular damage; consequently, there is little consensus on the underlying causality of the cellular damage.

Moreover, there is considerable controversy as to whether the parent compound or one of its metobolites is the cytotoxic agent. Some studies suggest that the major metabolites, daunorubicinol (DAUNol) and doxorubicinol (DOXol), are responsible for the adverse effects (Olson et al., 1988; Behnia and Boroujerdi, 1999; Forrest et al., 2000; Olson et al., 2003) while others claim that the parent drug is more toxic (Bernardini et al., 1991; Gonzalez et al., 1995; Ax et al., 2000; Veitch et al., 2009). These studies used a variety of different protocols and biological systems (different cell lines or animal models) to assess drug associated toxicity, and the differences among these various methodologies may underlie the disparity in the findings.

In this study we examined eight different human cell lines derived from different tissues and one rat embryonic cardiac cell line to evaluate toxicity of DAUN and DOX, or their alcohol metabolites, DAUNol, and DOXol. We included a heart cell line because the most serious side effects of anthracycline treatment are chronic cardiomyopathies, such as reduced LVEF and CHF and we wished to determine its sensitivity in comparison with other cell types. Our data consistently shows that the parent compounds, DOX and DAUN, and not their major metabolites, are the primary cytotoxic agents.

Two groups of enzymes have been implicated in the metabolism of DAUN and DOX; these are the aldo-keto reductases (AKRs) and carbonyl reductases (CBRs) (Cummings et al., 1991; Jin and Penning, 2007). We examined the relative abundance and enzymatic activity of eight AKRs and two CBRs in each of the nine cell lines, and found that the degree of cytotoxicity in each cell line is correlated with the activity of these enzymes and their ability to metabolize DAUN and DOX. The cells lines differed in their sensitivity to the drugs and the heart cell line was among the most sensitive to these anthracycline drugs. Four cell lines were more tolerant to the cytotoxic effects of the parent drug and this correlated with higher levels of enzyme activity and increased production of the major metabolite. These findings correlated with greater abundance of the AKRs and CBRs within the intracellular fluid of the drug-tolerant cell lines compared to the other five, drug-sensitive, cell lines. Finally, we showed that exposure to either drug results in an increase in AKR and CBR enzyme activity, and this increase in metabolic capacity correlates with an increase in the abundance of these enzymes.

MATERIALS AND METHODS

Chemicals and enzymes

Daunorubicin hydrochloride, doxorubicin hydrochloride, dexrazoxane (DEX) {4-[(2*S*)-2-(3,5-dioxopiperazin-1-yl)propyl]piperazine-2,6-dione}, dimethyl sulfoxide (DMSO), idarubicin hydrochloride (IDA) {(7*S*,9*S*)-9-acetyl-7-[(2*R*,4*S*,5*S*,6*S*)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-5,7,8,9,10,12-hexahydrotetracene-5,12-dione}, 100X antibiotic/antimycotic, potassium phosphate (KH_2PO_4), 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), N,N,N',N'-tetramethylethylenediamine, sodium chloride (NaCl),

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sodium monobasic phosphate (NaH_2PO_4), Tween® 20, and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO). Doxorubicinol (DOXol) and daunorubicinol (DAUNol) [chemical structures provided in Bains et al. (2008)] were obtained from SynFine Research (Richmond Hill, Ontario). High performance liquid chromatography (HPLC)-grade acetonitrile, ammonium persulfate ($(NH_4)_2S_2O_8$), formic acid (HCOOH), glycine (NH_2CH_2COOH), glycerol ($HOCH_2CH(OH)CH_2OH$), and Tris ($NH_2C(CH_2OH)_3$) were purchased from Thermo Fisher Scientific (Waltham, MA). Phosphate-buffered saline (PBS, pH 7.4), 100X antibiotic/antimycotic, and 0.25% trypsin were ordered from Invitrogen™ Corporation (Carlsbad, CA). Fetal bovine serum albumin (FBS) and 100X ProteaseArrest™ were supplied by the American Type Culture Collection (Manassas, VA) and G-Biosciences (Maryland Heights, MO), respectively.

Cell culture

The OVCAR-4 (ovarian) was kindly provided Dr. Thomas Hamilton (Fox Chase Cancer Center, Jenkintown, PA). The H9c2 rat heart embryonic cell line, as well as HepG2 (liver), NCI-H460 (lung), PANC-1 (pancreas), A-498 (kidney), MCF-7 (breast), PC-3 (prostate), and HCT-15 (colon) human carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). HepG2, A-498 and MCF-7 cells were cultured in Eagle's minimum essential medium. H9c2 and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium, while the PC-3 cells were cultured with F-12K medium. OVCAR-4, NCI-H460, and HCT-15 cells were maintained in RPMI-1640 medium. All cells were supplemented with 10% FBS and 1X antibiotic/antimycotic. Cells were grown on BD Falcon™ cell culture dishes (100 mm x 20

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mm; BD Biosciences, Mississauga, Ontario) and maintained in a humidified incubator with 5% CO_2 at 37°C.

MTT cell viability assay to measure cytotoxicity

Cells were seeded at densities of approximately 50-60% in Nunclon™ 48-well cell culture plates (Thermo Fisher Scientific) and were allowed to attach overnight in a humidified incubator with 5% CO_2 at 37°C. The next day, cells were treated with varying concentrations of DAUN or DOX (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10, 25, 50, 100, and 150 μM) for specified time periods (0, 6, 24, or 48 hrs). After treatment for each of the time periods, MTT solution [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 5 mg/mL in PBS, pH 7.4] was added (62.5 μl/well), and the plates were incubated for another 4 hrs at 37°C. Living cells are able to reduce MTT to an insoluble, colored (dark purple) formazan product by mitochondrial succinate dehydrogenase, while dead cells cannot. Following the 4 hr incubation period, cells were harvested and solubilized by DMSO and absorbance values of the formazan product were measured at 570 nm (37°C) using a monochromator-based Synergy™ M_x multi-mode microplate reader with Ultra Fine-Tuned™ performance. Absorbance values were recorded using the Gen5™ version 1.08.4 software program (BioTek Instruments, Inc., Winooski, VT). Vehicle-treated cells (water without drug) were used as controls. For each cell line exposed to DAUN or DOX at each time interval, MTT assays were performed in three independent experiments and repeated in triplicate. The percentage of live cells was calculated relative to the control wells using the following equation: [(Absorbance value of experimental well)/(Absorbance value of control well)] x 100. These percentage values were used to construct lethal concentration (LC_{50}) dose-response curves with the GraphPad Prism version 4.0 program (GraphPad Software Inc., San Diego, CA). LC_{50} values for each cell line were determined following DAUN and DOX

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exposure at each concentration of the drug and at the aforementioned time intervals. MTT assays were repeated again to determine LC_{50} values in all cell lines for the 48 hr exposure period with the major metabolites, DAUNol and DOXol (0, 0.5, 1, 5, 10, 25, 50, 100, 250, 500, 1000, 1500, 2000, and 3000 µM)**.**

Permeability assays to quantify extra- and intracellular anthracycline levels

Permeability studies were done to determine if there were differences among the nine cell types in their ability to absorb or export either the parent drug or its primary metabolite. For this set of experiments, cells were grown in BD Falcon™ cell culture dishes (35 mm x 10 mm) and maintained in a humidified incubator with 5% CO_2 at 37°C in their respective growth media until they achieved a cell density of 50%. Media was aspirated the following day, and replaced with 4 ml media with a final concentration of either 1 µM DOX or DOXol. With respect to anthracyclines such as DOX and DAUN, 1 µM represents human physiological concentration (Hempel et al., 2002; Liu et al., 2008; Hanna et al., 2011). For each cell line, different culture dishes were set up for incubation at 0, 20 mins, 1 hr, 3 hrs, 6 hrs, and 24 hrs. Following the incubation time period, the media (representing the extracellular portion) was collected and retained for further analyses. The cells were then washed twice with PBS (4 ml volume per wash) and collected in 0.5 ml PBS using a scraper; they were then lysed by sonication, and centrifuged at 10,000x*g* for 10 min at 4^{o}C to obtain the supernatant fraction containing the intracellular fluid (cytosol and nuclear fluid) while the pellet fraction containing cellular debris and genomic DNA was discarded.

The media collected from the permeability assays was diluted 15-fold in the same culture media to a final volume of 150 µl and placed into 1.5 ml Eppendorf tubes. The intracellular

samples were also diluted 15-fold to a final volume of 150 µl, except that the dilution was in PBS. To each of the tubes, 300 µl of ice-cold acetonitrile was added, which contained 0.4 µg/ml IDA as an internal standard. The media and intracellular samples were then subjected to vortex mixing and centrifuged at 10,000x*g* for 10 min at 4°C to remove protein. The supernatants were removed for each sample, and quantification of DOX and DOXol was achieved using LC/MS/MS.

DAUN and DOX *in vitro* metabolic assays

Intracellular fluid (cytosol and nuclear fluid) from the rat heart and human carcinoma cell lines were extracted according to the protocol provided for mammalian cells by QIAGEN Inc. (Mississauga, ON). Cells were washed in PBS, collected for 5 min at 1000x*g*, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) with 0.05% Tween® 20, and lysed by sonication. The lysate was centrifuged at 10,000x*g* for 10 min at 4°C to pellet cellular debris and genomic DNA. A final concentration of 1X ProteaseArrest™ was added to the supernatant to prevent protein degradation, and the supernatant was stored for metabolic assays in 20% glycerol at -20°C.

The enzymatic conversion of DAUN and DOX to their respective major metabolites, DAUNol and DOXol, were measured in intracellular fluid collected from cell lines. Assays were performed in 1.5 ml Eppendorf tubes, closely resembling to the Kassner et al. (2008) study: 20-30 µg total intracellular fluid protein, 1 µM DAUN or DOX substrate, 2 mM NADPH, 100 mM KH_2PO_4, pH 7.4 at 37°C, 30 min at 250 rpm. A vehicle control (water) was also run in conjunction with the metabolic assays. The total volume for each assay was 150 µl.

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Effect of DEX on DOX metabolism

Sometimes DEX is incorporated with anthracyclines as a potential cardioprotective agent during cancer treatment (Wouters et al., 2005; Choi et al., 2010). Therefore, another set of metabolic assays was performed with the addition of DEX, to determine whether it significantly altered the metabolic conversion of anthracyclines to their major metabolites. In North America, DEX is administered at 10-fold the DOX dose (10:1 ratio), therefore, 10 µM was chosen as a final concentration in the metabolic assays (Plandé et al., 2006). A vehicle control (water), along with a DEX only control (no DOX present), was performed in conjunction with the aforementioned metabolic assays.

Intracellular fluid protein concentrations used in the metabolic studies were determined using the Bradford protein assay with bovine serum albumin as a standard. The enzymatic reaction was stopped by adding 300 µl of ice-cold acetonitrile, which contained 0.4 µg/ml IDA as an internal standard, followed by vortex mixing and centrifuging at 10,000x*g* for 10 min at 4°C to remove protein. The supernatant was removed, and quantification of the DAUNol and DOXol metabolites was achieved using LC/MS/MS.

Instrumentation and experimental conditions for LC/MS/MS

The LC/MS/MS analytical method is based on a previously reported assay with modifications to the chromatography, sample preparation, and detection (Lachâtre et al., 2000). The metabolic assays were analyzed using an ultra-performance LC/MS/MS (UPLC/MS/MS) system, which consisted of a Waters Acquity ultra performance liquid chromatograph connected to a Waters Quattro Premier XE triple quadrupole mass spectrometer using an electrospray positive ionization source (Waters® Corporation, Milford, MA). Chromatographic separation of

DOX, DOXol, DAUN, DAUNol and IDA (internal standard) was performed using a Waters Acquity UPLC™ bridged ethyl hybrid C_{18} column (100 mm x 2.1 mm i.d., 1.7 µm particle size) with the following mobile phase composition and gradient programming: water with 0.1% formic acid (A), acetonitrile with 0.1% formic acid (B); 0 min, 5% B; 1 min, 5% B; 2 mins, 98% B; 4 mins, 98% B; 4.1 mins, 5% B; and 6 mins, 5% B. The flow rate was maintained at 0.2 ml/min with a total run time of 6 mins. The mobile phase flow was diverted to waste before 2.5 mins and after 3.5 mins during the chromatographic run. The mass spectrometer was operated in positive electrospray ionization mode with a capillary voltage of 3.5 kV, cone voltage of 40 V, and desolvation temperature of 300 ºC. The analytes were detected in multiple reaction monitoring (MRM) mode using the total ion current of the following ion transitions, and collision energy (CE, eV) values: for DOX *m/z* 544.1 → 361.2 (CE 20), *m/z* 544.1 → 379.0 (CE 20), and *m/z* 544.1 → 397.2 (CE 10); for DOXol *m/z* 546.0 → 345.0 (CE 35), *m/z* 546.0 → 363.1 (CE 20), and *m/z* 546.0 → 398.9 (CE 10); for DAUN *m/z* 527.9 → 321.0 (CE 20), *m/z* 527.9 → 363.2 (CE 20), and *m/z* 527.9 → 381.0 (CE 10), for DAUNol *m/z* 530.1 → 306.2 (CE 35), *m/z* 530.1 → 321.2 (CE 30), and *m/z* 530.1 → 383.1 (CE 10); and for IDA *m/z* 497.9 → 291.2 (CE 25), *m/z* 497.9 → 333.0 (CE 25). Data were acquired and processed using the MassLynx™ version 4.1 software (Waters® Corporation). The limit of quantitation for DOXol and DAUNol was 4.58 nM and 4.42 nM, respectively. Accuracy and precision met the acceptance criteria of less than 15% bias from the theoretical concentration and less than 15% coefficient of variation for calculated concentrations with six replicate samples prepared at three quality control levels within the range of the method.

For the permeability assay analyses, the UPLC/MS/MS method was transferred to a UHPLC/MS/MS system, and DOXol was quantified using this instrumentation. The

UHPLC/MS/MS system consisted of an Agilent 1290 Infinity Binary Pump, an Agilent 1290 Infinity Sampler, an Agilent 1290 Infinity Thermostated Column Compartment connected to an AB Sciex QTRAP® 5500 hybrid triple quadrupole trap mass spectrometer equipped with a TurboIonSpray® ionization probe. Chromatographic separation of DOX, DOXol, and IDA was achieved using the same column, mobile composition and flow rate as for the UPLC/MS/MS system, and the gradient programming was slightly modified as follows: 0 min, 10% B; 0.5 mins, 10% B; 4 mins, 90% B; 5 mins, 90% B; 5.1 mins, 10% B; and 7 mins, 10% B. The total run time was 7 mins, and the mobile phase flow was diverted to waste before 3.0 mins and after 5.0 mins during the chromatographic run.

The QTRAP® 5500 mass spectrometer was operated in positive ionization mode with a curtain gas flow of 30 units, ion spray voltage of 5500 V, TurboIonSpray® temperature of 300 ºC, and collision-activated dissociation (CAD) set high. DOX, DOXol, and IDA were detected in MRM mode, at unit resolution, using similar ion transitions as described for the UPLC/MS/MS method. Data were acquired and processed using the Analyst version 1.5.2 software (AB Sciex LP). The UHPLC method was qualified for the quantification of DOX and DOXol. The limit of quantification for DOX and DOXol were improved to 1.72 nM and 1.83 nM, respectively. Accuracy and precision met the acceptance criteria of not more than 16% bias from the theoretical concentration, and not more than 12% coefficient of variation for calculated concentrations with four replicate samples prepared at three quality control levels within the range of the method.

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Western blotting of intracellular fluid from cell lines to detect AKRs and CBRs

Western blot analyses of the intracellular fluid extracted from each cell line were conducted according to the manufacturer's instructions for the Odyssey™ (LI-COR Biosciences, Lincoln, NE). Following 18% SDS-polyacrylamide gel electrophoresis, intracellular proteins were transferred at 20 V in Towbin's buffer (25 mM Tris, 192 mM glycine, and 20% v/v methanol) overnight (at 4°C) to a Hybond-C Extra nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membranes were blocked in Odyssey blocking buffer, and the enzyme was detected using either a MaxPab polyclonal mouse anti-human AKR1A1, AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR1C4, AKR7A2, CBR1 or CBR3 antibody (Abnova® Corporation, Taipei City, Taiwan) (diluted 1:2500) as the primary antibodies and IRDye 800CW goat anti-mouse IgG as the secondary antibody (diluted 1:5000) (LI-COR). Previous studies suggest that the reductase enzymes metabolize DAUN and/or DOX; hence, they were selected for this study (Ohara et al., 1995; O'Connor et al., 1999; Martin et al., 2006; Kassner et al., 2008; Bains et al., 2008; Bains et al., 2009; Bains et al., 2010a). Both primary and secondary antibodies were in blocking buffer containing 0.1% Tween 20. The bound secondary antibody was detected using the Odyssey™ Infrared Imaging system (LI-COR). Beta-tubulin served as a loading control and was detected using the same protocol, except that a mouse monoclonal anti-beta-tubulin primary antibody (Abcam® Inc., Cambridge, MA) was used (diluted 1:1000). Band intensities for AKR or CBR proteins were determined using Odyssey™ densitometry software, and were normalized to band intensities of beta-tubulin in order to quantify the relative expression of the individual reductases in each intracellular fluid extract.

Induction of AKR and CBR expression in cell lines exposed to anthracycline drugs

All the cell lines were pre-treated with 100 nM of either DAUN or DOX for 0, 6, 24, or 48 hrs. This drug concentration was selected since cell viability was 75% or greater based on the dose-response curves generated from the cytotoxicity study. For analysis at each time interval, the cells were washed three times with PBS, intracellular fluid extracts prepared, and tested for their ability to metabolize DAUN or DOX to their respective major metabolites, DAUNol and DOXol. The *in vitro* metabolic assays were conducted as described above employing either 1 μM DAUN or 1 μM DOX as substrates.

We performed Western blots on intracellular fluid extracts from cells exposed to DOX and DAUN to determine whether increases in drug metabolic activity correlated with increased expression (protein levels) of some or all of the AKRs and CBRs analysed in this study. The relative abundance of each of the reductase enzymes, in each intracellular fluid extract was determined as previously described (see Western blotting of cell line extracts).

Statistical analysis

Statistical analyses were performed using GraphPad Instat® (version 3.6; GraphPad Software Inc., San Diego, CA). Percent viabilities used to create dose-response curves from the cytotoxicity assays were expressed as means ± S.D., while LC_{50} values were provided along with the 95% confidence intervals. Results from the permeability/metabolic assays and reductase expression studies were reported as means ± S.D., and compared using one-way analysis of variance followed by Tukey-Kramer multiple comparisons tests. Differences were considered significant at $p<0.05$.

RESULTS

Anthracycline cytotoxicity

The cytotoxicity of the anticancer drugs DAUN, DOX, and their metabolites (DAUNol and DOXol) were determined in nine different cell types using the MTT assay. Cell viability was measured after incubating each cell line with various concentrations of the anthracycline parent drug or its major alcohol metabolite for 0 (100% viable), 6, 24, or 48 hrs. Dose-response curves were plotted for all nine cell lines; two representative plots are presented in Supplemental Fig. 1. The LC_{50} values for all nine cell types were calculated from these curves and are provided in Tables 1 (for DAUN and DAUNol) and 2 (for DOX and DAUNol). Three observations are worth mentioning from these data sets. First, the nine different cell types were easily grouped into two categories. Four cell lines, HepG2 (liver), HCT-15 (colon), NCI-H460 (lung), and A-498 (kidney), were found to be more tolerant to DAUN and DOX (hereafter called the drug-tolerant group) compared to the five remaining cell lines, H9c2 (heart), PC-3 (prostate), OVCAR-4 (ovary), PANC-1 (pancreas), and MCF-7 (breast), (hereafter called the drug-sensitive group). This finding was consistent for all time points examined, and was statistically significant in all cases. Second, DOX was significantly more toxic than DAUN for each of the cell lines tested. Third, in all nine cell types, DAUNol and DOXol were significantly less toxic than their respective parent drug. Indeed, the DAUNol LC_{50} values were 30- to 108-fold greater than those for DAUN while the DOXol LC_{50} values were 59- to 162-fold greater than those for DOX. Taken together, the simplest interpretation of these findings is that the parent drugs, DAUN or DOX, are substantially more toxic to these cell lines than are their metabolites, DAUNol and DOXol, respectively.

Nonetheless, the alternative hypothesis, that the metabolites are more toxic than the parent drug and that toxic concentrations of the metabolites are generated by the metabolism of the parent drug, is also possible. However, this hypothesis necessitates one of two stipulations: either the drug-sensitive group must metabolize the parent compound more efficiently than the drug-tolerant group, or those experiments in which the cells were exposed the alcohol metabolites (not the drugs), the uptake of the metabolites must be lower in the drug-tolerant group than in the drug-sensitive group, resulting in lower intracellular concentrations in the drug-tolerant group.

Permeability of cell lines to DOX and DOXol

We performed permeability assays to determine whether differences in rate of drug or metabolite uptake and/or efflux were associated with the substantial difference in LC_{50} values between the drug-tolerant and drug-sensitive cell types. A sub-set of the cell lines from the drug-tolerant (HepG2, A-498 and Hct-15) and drug-sensitive (H9c2, PANC-1 and MCF-7) groups were used in these assays. Each cell line was exposed to either DOX or DOXol for periods of 20 mins, 1 hr, 3 hrs, 6 hrs and 24 hrs. After the treatment period, the media and cells were separated as described (see Materials and Methods) and the amount of extracellular (in media) and intracellular (cytosolic and nuclear fluid) parent drug and its major metabolite were determined.

Four observations can be made from the permeability studies. First, during the 24 hr incubation period, cell lines exposed to 1 μM DOX exhibited significantly greater intracellular DOX levels (Table 3A) compared to intracellular DOXol levels from the same cell lines exposed to 1 μM DOXol (Table 3B). Although DOX and DOXol are structurally similar, the difference in the hydroxyl group on carbon-13 of DOXol likely hinders its influx compared to DOX.

Second, the intracellular levels of DOXol were nearly identical regardless of whether the cells were exposed to either 1 μM DOX or DOXol during the treatment periods (compare Tables 3B and 3C). Therefore, it is unlikely that the accumulation of the major metabolite, DOXol, plays a significant role in anthracycline-associated cytotoxicity. Third, following 24 hrs of exposure to DOX, the amount of intracellular DOXol (Table 3C) is significantly greater among the drug-tolerant cell lines (kidney, colon, liver) than it is among the drug-sensitive cell lines (heart, pancreas, breast). This observation, along with the observation that the intracellular levels of DOX are significantly higher among the drug-sensitive cells than among the drug-tolerant cells (Table 3A), suggest that both cell types are capable of taking up DOX, perhaps at relatively comparable rates, but that the drug-tolerant cells convert DOX to DOXol at a higher rate. Indeed, this correlates with the findings from the metabolic studies (see below). Moreover, the extracellular levels of DOXol are at least 3-fold higher than the intracellular levels in the cells treated with the parent drug (compare Tables 3C and 3D), which suggests that all of the cells are able to export the metabolite and the amount exported correlates with the amount generated. Fourth, the intracellular levels of DOX found in the three drug-tolerant cell lines and the three drug-sensitive cell lines are not significantly different from each other after 20 mins of exposure to DOX (Table 3A). However, after 24 hrs of exposure, the amount of intracellular DOX in the three drug-sensitive cell lines is significantly higher than that found in the three drug-tolerant cell lines**.** Taken together, these data suggest that the drug-tolerant cell lines convert DOX to DOXol more rapidly than the drug-sensitive cell lines. The alternative hypothesis, that the drug-tolerant cell lines take up DOX less efficiently, seems less likely since after a 20 min exposure period both cell types have taken up nearly identical amounts of DOX, and transport of the

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anthracyclines is by passive diffusion of the non-ionized free base, which is in equilibrium with the ionized drug (Skovsgaard and Nissen, 1982; Sparreboom et al., 2002).

In summary, the most parsimonious interpretations of these data are that the cell types do not show any notable differences in influx of the drug or efflux of its primary metabolite. The data suggest that the drug-tolerant cell lines metabolize DOX at higher rates than the drug-sensitive cell lines.

***In vitro* metabolic assays and Western Blot analyses of cell lines**

If the drug-tolerant group of cell types does metabolize DOX and DAUN more efficiently than the cell lines comprising the drug-sensitive group, then one would predict that they would have higher levels of the enzymes that metabolize these anthracyclines. Therefore, we (i) measured the amount of eight AKRs and two CBRs in each of the cell lines, and (ii) performed *in vitro* metabolic assays using cell line intracellular fluids with either DOX or DAUN as substrate and asked whether metabolic conversion of the drug to its metabolite is correlated with the enzyme levels and if either or both of these parameters correlate with the sensitivity of each cell type to these anthracycline drugs.

Among the five cell lines that comprise the drug-sensitive group (H9c2, PC-3, OVCAR-4, PANC-1, and MCF-7), the rates of conversion of DAUN to DAUNol ranged from 17.6±1.0 to 27.3±1.9 pmol DAUNol/min•mg intracellular fluid protein and the rate of conversion of DOX to DOXol ranged from 3.6±1.0 to 5.0±0.8 pmol DOXol/min•mg intracellular fluid protein (Fig. 1). The four cell lines that comprise the drug-tolerant group (HepG2, HCT-15, NCI-H460, and A-498) had significantly higher metabolic rates, ranging from 50.0±3.2 to 80.7±9.7 pmol DAUNol/min•mg and 9.3±0.8 to 10.5±1.1 pmol DOXol/min•mg (Fig. 1). As a result, the five

drug-sensitive cell lines were classified as “slow metabolizers” and the four drug-tolerant cell lines as “rapid metabolizers” of these anthracyclines. In addition, all cell lines were shown to metabolize DAUN more rapidly than DOX.

Western blot analysis of intracellular fluid fractions revealed that each of the “rapid metabolizer” cell lines express the eight AKRs and two CBRs at significantly higher levels than any of the “slow metabolizer” cell lines (Fig. 2). Distinct bands with electrophoretic mobilities corresponding to the calculated molecular mass for each of the enzymes, 35-38 kDa for the AKRs and 30 kDa for the CBRs, were detected in each extract (Supplemental Fig. 2). These data suggest that the difference between the “rapid” and “slow” metabolizing cell types in their rates of conversion of DAUN/DOX to DAUNol/DOXol in the *in vitro* assays correlate with the relative abundance of the AKR and CBR enzymes in the drug-tolerant and drug-sensitive cell lines.

Effect of DEX on *in vitro* metabolism of anthrayclines

DEX is a potent intracellular chelating agent, and as such it is thought to reduce the number of metal ions complexed with anthracyclines such as DOX, and consequently decrease the formation of superoxide radicals (Jones, 2008). Therefore, it is sometimes used with DOX treatment. However, the European Medicines Agency and the U.S. Food and Drug Administration stated in June and July 2011, respectively, the use of DEX should be restricted to adult patients with breast cancer who have received >300mg/m^2 DOX, and general approval for use for cardioprotection was withdrawn. Nonetheless, since DEX is sometimes still used with DOX in breast cancer treatment, we decided to determine whether DEX significantly altered the metabolic conversion of DOX to DOXol. These assays were performed using intracellular fluid

fractions of three of the DOX-tolerant cell lines (HepG2, HCT-15, and NCI-H460) and three of the DOX-sensitive cell lines (H9c2, PANC-1, and MCF-7). The results showed no significant effect of DEX on DOX metabolism (data not shown). Hence, if DEX does have a cardioprotective affect, it doesn't seem to occur by impacting the primary conversion of DOX to DOXol.

Induction of AKR and CBR enzymes in cell lines following exposure to DOX or DAUN

Next, we asked whether exposure to either DAUN or DOX induced an increase in the abundance of the AKR or CBR enzymes in one or more of the nine cell lines. In addition, if exposure to DAUN or DOX did elicit a response, then we wanted to determine whether the drug-tolerant and drug-sensitive cell types differed in their response. The cells were exposed to DOX or DAUN for 0, 6, 24, and 48 hr time periods; they were then removed from the drug containing media and, following several washes in PBS, the cells were lysed and their ability to metabolize DOX or DAUN was compared. Finally, in order to determine whether any alteration in the metabolism of the drugs was correlated with induction, we measured the relative abundance of each enzyme in the cell lines following exposure to the drug for the each of time periods.

As seen in Fig. 3, after a 6 hr exposure to the drug, all nine cell lines exposed to DOX, and eight of the nine cell lines exposed to DAUN (the exception is the lung cell line), had an observable increase in the activity of the AKR and CBR enzymes; however, while the increase in metabolic activity was consistently observed and easily quantified, it was not statistically significant. Statistically significant increases in DAUN/ DOX metabolism were observed after 24 hrs and even greater increases were observed among the cells exposed for 48 hrs. Indeed, all nine cell types responded similarly; they all up-regulated their ability to metabolize DAUN or

DOX. There was a 1.4- to 2.2-fold increase in DAUNol and 1.8- to 2.0-fold increase in DOXol production in all nine cell types after 24 and 48 hrs of exposure to DAUN and DOX, respectively. There was no difference in the relative magnitude of induction of the AKR and CBR enzymes in response to DAUN or DOX exposure between the cells comprising the drug-tolerant group and those comprising the drug-sensitive group. However, since the drug-tolerant group had a significantly higher initial ability to metabolize the anthracyclines, and the level of induction of anthracycline metabolizing enzymes is approximately the same in all cell lines, the drug-tolerant cell lines maintain their higher capacity for the metabolism of DOX and DAUN after exposure to these drugs.

Undoubtedly, the induction of enzyme activity required some time, since after a 6 hr exposure to either drug the increase in metabolic activity was unequivocally evident but not statistically significant. This time interval accords with the time required for transcription, translation, and accrual of the AKR and CBR enzymes. Hence, it is reasonable to speculate that the increase in metabolic activity in response to exposure to DAUN or DOX is at least partially caused by the increase in the amount of the various AKR and/or CBR enzymes. To test this hypothesis, Western blot analyses was used to examine the relative abundance of two enzymes (AKR1C3 and CBR1) in each of the nine cell lines exposed to DAUN or DOX for 0, 6, 24, or 48 hrs. These two enzymes were chosen because our previous *in vitro* studies demonstrated that AKR1C3 and CBR1 have the highest catalytic efficiency (k_{cat}/K_m) towards DOX and DAUN, respectively, compared to the eight remaining AKR and CBR enzymes (Bains et al., 2010b). The Western blot analyses (Fig. 4) demonstrated that the relative abundance of AKR1C3 and CBR1 increased over time in all nine cell lines exposed to either DAUN or DOX. An easily quantified, but non-statistically significant, increase in these enzyme levels was observed after 6

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hrs. After 24 hrs of exposure, all cell lines showed statistically significant increases in the abundance of AKR1C3 and CBR1. For AKR1C3, significant increases in expression were detected in both the 24 and 48 hr DAUN-treated (1.2- to 2.3-fold increase compared to non-treated cell lines) and DOX-treated (1.2- to 2.7-fold increase) cell lines. There was a comparable increase in CBR1 expression among cells exposed for 24 and 48 hr to either DAUN (1.3- to 2.8-fold increase) or DOX (1.5- to 2.4-fold increase). With each of the cell lines, whether pre-treated for 6, 24, or 48 hrs, plotting the increase in both DAUN/DOX metabolic activities against the abundance of AKR1C3/CBR1 revealed a strong association between these variables [coefficients of determination (r^2) were ≥ 0.80]. Thus, the induction in AKRs and CBRs appears to be the major source for the increase in DAUN and DOX metabolism observed among cell lines exposed to these drugs versus their untreated counterparts. Previous studies have demonstrated significant induction of AKRs and CBRs in the presence of DOX. For example, exposure of MCF-7 breast tumor cells to low (nanomolar) concentrations of DOX resulted in a significant increase in CBR1 protein levels (Gavelová et al., 2008), as well as AKR1C2 and AKR1C3 (Veitch et al., 2009). In relation to DAUN, the current study was the first to demonstrate that exposure to this anthracycline causes a significant induction in the expression of the CBR1 and AKR1C3 enzymes, and to show that this induction occurs in a wide variety of cell types.

Association between cytotoxicity and DOX/DAUN metabolism in cell lines exposed to these anthracyclines

For each of the incubation time periods, the LC_{50} values (DAUN and DOX) from the cytotoxicity studies were logged and plotted against the metabolic activity rates from the DAUN and DOX-treated cell lines to see if an association exists between these two variables (Fig. 5).

The high values obtained for correlation coefficients (r) and coefficients of determination (r^2) suggest that anthracycline-induced cytotoxicity and metabolism show a strong, inverse correlation.

DISCUSSION

DAUN and DOX are commonly used to treat a variety of cancers, but there is considerable inter-patient variability in both efficacy and toxicity of these drugs. One hypothesis is that this variability arises from the amount of drug metabolized and consequently eliminated from the body. There is no conclusive evidence linking altered DAUN or DOX metabolism to toxicity, and the argument is further encumbered by conflicting assertions of whether cytotoxicity is caused by the parent drug or its major metabolite. To address these issues, we performed *in vitro* studies on nine different cell lines to determine: (i) whether the parent drugs, DAUN or DOX, or their major metabolites, DAUNol or DOXol, respectively, are more cytotoxic, (ii) the efficiency with which each cell line metabolizes the parent drug to its major metabolite, and (iii) whether the metabolic capability of each cell line correlates with the relative abundance (expression) of AKR and CBR enzymes.

The findings from the cytotoxicity studies with the parent drugs led to the allocation of nine cell lines into two distinct sub-groups, drug-sensitive and drug-tolerant. The drug-sensitive group comprises of five cell lines (heart, prostate, ovary, pancreas, and breast) due to their significantly higher LC_{50} values compared to the four remaining cell lines (liver, colon, lung, and kidney), which encompasses the drug-tolerant group (Tables 1 and 2). LC_{50} values also

indicated that the major metabolites are significantly less cytotoxic than their respective parent drugs.

One could argue that these differences in cytotoxicity are due to the accumulation or efflux of the parent drugs or major metabolites. Permeability assays were performed to measure intracellular DOX and DOXol levels of three drug-tolerant and drug-sensitive cell lines following their exposure to either compound for varying lengths of time. DAUN and DAUNol were not used since these compounds would likely yield similar findings because of similarities in chemical structure to DOX and DOXol. The intracellular concentration of DOX was significantly higher than that of DOXol among all six cell lines that were exposed to DOX versus DOXol, despite duration of exposure (compare Tables 3A and 3B). All six cell lines tend to accumulate DOX with similar efficiency, as demonstrated with the intracellular levels following 20 mins of exposure to the parent drug (Table 3A). As exposure time increased to 24 hrs, intracellular DOX levels among the drug-sensitive cell types were significantly higher compared to the drug-tolerant cell types. Moreover, higher intracellular DOXol levels were seen among all six cell lines as exposure duration increased regardless of whether DOXol was generated by metabolism (cells exposed to the parent drug, Table 3C) or by influx of DOXol (cells exposed to the metabolite, Table 3B). Although intracellular levels of DOXol were measured, extracellular levels were found to be least three times higher (compare Tables 3C and 3D), suggesting that efflux of this metabolite occurs readily, and does not limit its accumulation in the cytosol or nuclear fluid. In addition, intracellular and extracellular DOXol levels were significantly higher with the drug-tolerant cell lines versus the drug-sensitive cell lines after 6 and 24 hrs of parent drug exposure. Overall, the cytotoxicity and permeability data provide considerable evidence that the parent drug is likely more cytotoxic than the major metabolite.

Since previous studies revealed DOXol or DAUNol as the major metabolites in cancer patients receiving treatment with DOX or DAUN, respectively (Lipp and Bokemeyer, 1999; Plebuch et al., 2007), we assessed the efficiency with which all nine cell lines metabolize DOX and DAUN by quantifying the rate at which the major metabolites were produced. Clearly, all nine cell types were able to metabolize either DAUN or DOX, but they differed in the rate at which they converted the parent drug to its major metabolite. The four drug-tolerant cell types metabolized DAUN or DOX faster than the five drug-sensitive cell types. The classification of cells into drug-tolerant versus drug-sensitive groups based on cytotoxicity studies correlates very well with their metabolic efficiency (correlation coefficients range from about 0.92 to 0.97, depending on exposure times, with correspondingly robust coefficients of determination). Thus, there is a very strong association between these two physiological phenomena.

Finally, the relative abundance of AKR and CBR enzymes in the nine different cell lines correlated with the relative efficiency with which each cell type metabolized DAUN or DOX. The cell lines that metabolized the drugs rapidly had considerably more intracellular AKR and CBR enzyme (often 2- to 3-fold greater) than the amount present in the cell types that metabolized the drugs slowly. Therefore, there is a striking parallel between the efficiency with which each cell type metabolizes DAUN or DOX and the relative abundance of the anthracycline metabolizing enzymes. The drug-tolerant cell lines metabolized DAUN or DOX more quickly and they exhibited higher abundance of AKR and CBR enzymes, while the reverse is true for the drug-sensitive cell lines. When this data is taken together with the permeability data, it suggests that the difference between the drug-tolerant and drug-sensitive cell types is the rate at which they metabolize the parent drugs, DAUN and DOX. When paired with the cytotoxicity data,

they provide compelling evidence that DAUN and DOX are more cytotoxic than their major metabolites.

Some caveats should be stated. First, the cells used are permanent cell lines derived from human or rat tissues. As immortalized cells, their growth properties have been altered; yet, we reckon that these cell lines retain most of the biochemical distinctiveness of their respective parent cell types. Second, although there is a striking inverse relationship between the relative abundance of the selected AKR/CBR enzymes and the sensitivity of each cell line to DAUN/DOX-induced cytotoxicity, the findings do not establish a direct involvement of any of these reductase enzymes with drug sensitivity. Nonetheless, we believe the results provide a compelling reason for additional studies to determine the basis for the correlation between AKR and CBR abundance and cytotoxicity following exposure to these anthracycline drugs. Future studies should include (i) inhibiting or reducing the metabolic activity of AKRs and CBRs (via knockdown/knockout procedures, or the use of specific inhibitors) in cell lines with high levels of reductase expression and DAUN/DOX metabolic activity to determine whether this increases the cell's sensitivity to the anthracycline drugs, and (ii) over-expressing the individual AKRs and CBRs (via transfection with expression vectors containing the reductase gene of interest) in cell lines with low levels of reductase expression/metabolic activity to see if this decreases the cell's sensitivity to the drugs. A couple of studies of this sort have been performed. For example, Tak et al. (2011) demonstrated that overexpression of human CBR1 in hepatocellular carcinoma cells enhanced cell survival by decreasing oxidative stress under DOX treatment. Another study found that inhibiting AKR1C with 5 beta-cholanic acid in ABCB1-deficient cells, that had become DOX-resistant, restored their sensitivity to DOX while having no effect on an ABCB1-expressing epirubicin-resistant cell line (Veitch et al., 2009). For the remaining AKRs and

CBRs, there are currently no published studies that examine the effects that altering their expression levels may have on cell viability in the presence of either DAUN or DOX.

Overall, our study demonstrates an inverse association between DAUN/DOX metabolism by AKRs/CBRs and cytotoxicity using nine cell lines. If this cytotoxicity is mechanistically similar to the toxicity observed following anthracycline therapy in cancer patients, then one would expect that the relative toxicities among the nine cell lines would parallel the relative sensitivity of normal cells in the tissue types from which the cell lines were derived. The heart cell line was the most sensitive to either DAUN or DOX, while the liver cell line was the most tolerant. This difference correlates with the abundance of the AKR and CBR enzymes which is lower in heart tissue than liver (Bains et al. 2010b). This is not unexpected since these anthracyline drugs are processed primarily in the liver. A study by O'Connor et al. (1999), using Western blot analyses, demonstrated that AKR7A2 and AKR1B1 are also expressed in the human heart; unfortunately, they did not quantify the relative abundances of these two enzymes.

Given the correlation between the efficiency with which each cell line metabolizes DAUN or DOX, and their sensitivity to the cytotoxic effects of these drugs, one might predict that cancer patients with mutant alleles of AKR and CBR genes that reduce enzyme function are more likely to develop toxicities following DAUN or DOX therapy. Clinical studies the compare patients with natural variants in one or more of these AKR and CBR genes to those with normal alleles are needed to determine if one or more of these variants are clinically relevant genetic biomarkers for guiding anthracycline therapy. Very recently, a variant of CBR3 was shown to be associated with changes in short-term functional cardiac parameters in patients who have undergone anthracycline therapy (Volkan-Salanci et al., 2012). However, no association was found between DAUN therapy and cardiotoxicity in a small study of 13 naturally

occurring non-synonymous single nucleotide polymorphisms in the AKRs and CBRs of 185 cancer patients (Lubieniecka, et al., 2012).

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Bains, Szeitz, Lubieniecka, Cragg, Grigliatti, Reid, and Riggs

Conducted experiments: Bains, Cragg and Szeitz

Contributed new reagents and analytic tools: Szeitz

Performed data analysis: Bains, Cragg, Szeitz and Grigliatti

Wrote or contributed to the writing of the manuscript: Bains, Szeitz, Lubieniecka, Grigliatti, Reid, and Riggs

Other: Riggs, Reid and Grigliatti acquired funding for the research

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1—Enzymatic activities from the intracellular fluid of the rat and human cell lines incubated with 1 μM of daunorubicin (A) and doxorubicin (B). Activities were measured by following the rate of daunorubicinol and doxorubicinol production. Three independent batches of cells for each cell line were grown and intracellular fluid (cytosol and nuclear fluid) extracted. Assays were performed in quadruplicate with each batch. Enzymatic activities are reported as mean ± S.D. (n=12). Cell lines to the left of the vertical dotted line are sensitive to DAUN and DOX while cell lines to the right are the tolerant ones.

Fig. 2—Relative abundance of eight AKR and two CBR enzymes in the intracellular fluid of the five drug-sensitive [H9c2 (heart), PC-3 (prostate), OVCAR-4 (ovary), PANC-1 (pancreas), and MCF-7 (breast)] and the four drug-tolerant [(HepG2 (liver), HCT-15 (colon), NCI-H460 (lung), and A-498 (kidney)] cell types. Densitometry was used to determine the abundance of each reductase in each cell line following Western blotting. The densitometry value for each reductase protein was normalized to the level of beta-tubulin within that cell type in order to determine the relative abundance of each reductase in each cell type. Three independent batches of cells for each cell line were grown and subjected to Western blotting and densitometry analyses. Relative expression values are reported as mean ± S.D. (n=3). Cell lines to the left of the vertical dotted line are sensitive to DAUN and DOX while the cell lines to the right are the tolerant ones.

Fig. 3—The metabolic activity levels (measured as pmol DAUNol or DOXol/min•mg protein) among the intracellular fluid of each of the cell lines exposed to with 100 nM DAUN (A) or DOX (B) for 0, 6, 24, and 48 hrs time periods. Significant increases (*, p<0.05) in activity rates

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(following incubation with 1 μM of DAUN or DOX) are seen for the intracellular fluids treated for 24 and 48 hrs. Metabolic activity rates on the left side of the dotted vertical line represent the sensitive cell lines while activities to the right are from the tolerant cell lines. Three independent batches of cells for each cell line were grown and intracellular fluids extracted. Assays were performed in quadruplicate with each batch. Enzymatic activities are reported as mean ± S.D. (n=12).

Fig. 4—Sample Western blot analyses (A) of intracellular fluids from H9c2 rat heart and HCT-15 human colon carcinoma cell lines for purposes of assessing induction of AKR1C3 and CBR1 after 0, 6, 24, and 48 hr exposure to either 100 nM DAUN or DOX. Densitometry was performed on the immunoreactive bands and normalized against beta-tubulin (loading control) in order to calculate relative expression levels. The relative expression levels for AKR1C3 (B: DAUN-treated; C: DOX-treated) and CBR1 (D: DAUN-treated; E: DOX-treated) after pre-treatment with the drug; the asterisk indicates significant induction (*, p<0.05) in intracellular fluids treated for 24 and 48 hrs. Data shown on the left side of the dotted vertical line represent the relative abundance of the enzymes among the drug-sensitive cell lines, while data on the right are the levels found among the drug-tolerant cell lines. Three independent batches of cells for each cell line were grown and subjected to Western blotting and densitometry analyses. Relative expression values are reported as mean ± S.D. (n=3).

Fig. 5—Scatterplot of cytotoxicity ($\log_{10} LC_{50}$ values) and metabolic activity following DAUN (A) and DOX (B) treatment in the nine cell lines for the purposes of seeing if there is an association between these two variables. Low $\log_{10} LC_{50}$ values are synonymous with higher

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cytotoxicity, while the reverse is true for high $\log_{10} LC_{50}$ values. The high correlation coefficient (r) and coefficient of determination (r^2) values provided for each of the exposure time periods suggest a strong correlation between cytotoxicity and metabolism.

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TABLES

TABLE 1—Mean LC_{50} values of DAUN and DAUNol for cell lines at specified time intervals following exposure to the drug. LC_{50} values were calculated from dose-response curves generated from MTT viability experiments. 95% confidence intervals (CIs) are provided in brackets below the mean LC_{50} values. The first five entries of the table refer to the sensitive cell lines while the last four entries refer to the tolerant cell lines.

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CELL LINES	MEAN LC_{50} (μM) (95% CI)			
	DAUN 6 hrs	**DAUN 24 hrs**	**DAUN 48 hrs**	**DAUNol 48 hrs**
H9c2 (heart)	12.6 (11.2-14.1)	3.2 (3.0-3.5)	0.4 (0.3-0.5)	42.7 (37.6-48.5)
PC-3 (prostate)	10.2 (9.2-11.4)	3.2 (2.9-3.6)	0.4 (0.3-0.5)	43.2 (38.1-49.1)
OVCAR-4 (ovary)	11.1 (9.8-12.5)	3.1 (2.8-3.4)	0.7 (0.6-0.8)	41.6 (35.5-48.9)
PANC-1 (pancreas)	11.4 (10.1-13.0)	3.6 (3.2-4.0)	0.6 (0.5-0.7)	45.0 (39.5-51.4)
MCF-7 (breast)	14.1 (11.9-16.6)	4.2 (3.8-4.6)	0.6 (0.5-0.7)	48.7 (42.6-55.7)
Hep G2 (liver)	21.5 (18.4-25.1)	9.5 (8.3-10.9)	1.5 (1.3-1.9)	54.5 (47.8-62.0)
HCT-15 (colon)	19.7 (17.0-22.7)	9.9 (8.7-11.3)	1.4 (1.2-1.7)	54.0 (47.7-61.1)
NCI-H460 (lung)	21.0 (18.1-24.3)	10.3 (9.3-11.5)	1.7 (1.4-2.0)	55.0 (48.8-62.0)
A-498 (kidney)	22.9 (20.5-25.6)	13.7 (12.4-15.0)	1.7 (1.5-1.9)	51.7 (45.3-59.2)

TABLE 2—Mean LC_{50} values of DOX and DOXol for cell lines at specified time intervals following exposure to the drug. LC_{50} values were calculated from dose-response curves generated from MTT viability experiments. 95% confidence intervals (CIs) are provided in brackets below the mean LC_{50} values. The first five entries of the table refer to the sensitive cell lines while the last four entries refer to the tolerant cell lines.

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CELL LINES	MEAN LC_{50} (µM) (95% CI)			
	DOX 6 hrs	**DOX 24 hrs**	**DOX 48 hrs**	**DOXol 48 hrs**
H9c2 (heart)	5.1 (4.4-5.8)	1.1 (1.0-1.3)	0.2 (0.1-0.3)	25.8 (22.5-29.5)
PC-3 (prostate)	5.1 (4.6-5.7)	0.9 (0.8-1.1)	0.2 (0.1-0.3)	27.0 (23.2-31.5)
OVCAR-4 (ovary)	6.6 (5.9-7.5)	1.1 (0.9-1.2)	0.2 (0.1-0.3)	27.2 (24.0-30.7)
PANC-1 (pancreas)	7.4 (6.3-8.6)	1.3 (1.1-1.5)	0.2 (0.1-0.3)	29.6 (25.6-34.1)
MCF-7 (breast)	7.3 (6.4-8.3)	1.3 (1.1-1.5)	0.2 (0.1-0.3)	32.4 (28.2-37.1)
Hep G2 (liver)	11.9 (10.1-13.8)	3.4 (3.0-3.9)	0.5 (0.4-0.6)	37.2 (30.2-45.6)
HCT-15 (colon)	10.1 (9.1-11.1)	2.4 (2.2-2.6)	0.5 (0.4-0.6)	38.8 (34.8-43.3)
NCI-H460 (lung)	10.9 (9.8-12.0)	3.0 (2.6-3.4)	0.6 (0.5-0.7)	35.5 (30.7-41.0)
A-498 (kidney)	12.2 (10.6-14.0)	4.1 (3.7-4.6)	0.5 (0.4-0.6)	38.9 (34.4-44.0)

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TABLE 3—Anthracycline levels (in picomoles) from permeability assays using three cell lines from both the drug-sensitive and the drug-tolerant groups (sensitive: H9c2, PANC-1, and MCF-7; tolerant: A-498, HCT-15 and HepG2) treated 1μM parent drug (DOX) or major metabolite (DOXol) at intervals of 20, 60, 180, 360 and 1440 mins. (A) Intracellular DOX levels after treatment with DOX. (B) Intracellular DOXol levels after treatment with DOXol. (C) Intracellular DOXol levels after treatment with DOX. (D) Extracellular DOXol levels after treatment with DOX. DOX and DOXol levels are reported as mean ± S.D. (*n*=3).

(A)

	INTRACELLULAR DOX (pmol) from DOX incubation					
TIME (min)	**H9c2 (heart)**	**PANC-1 (pancreas)**	**MCF-7 (breast)**	**A-498 (kidney)**	**HCT-15 (colon)**	**HepG2 (liver)**
20	122±13	105±9	155±19	112±11	137±9	119±9
60	197±18	182±15	283±18[acd]	186±12	174±10	144±10
180	420±30[a]	316±29	384±14[a]	299±18	250±17	277±19
360	569±31[a]	467±40	595±25[a]	440±33	409±27	395±29
1440	748±38[a]	683±25[a]	783±45[a]	563±35	530±39	588±21

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(B)

	INTRACELLULAR DOXol (pmol) from DOXol incubation					
TIME (min)	**H9c2 (heart)**	**PANC-1 (pancreas)**	**MCF-7 (breast)**	**A-498 (kidney)**	**HCT-15 (colon)**	**HepG2 (liver)**
20	4±1	6±1	6±1	8±1[c]	6±1	8±1[c]
60	8±1	11±2	14±2	13±2	13±1[c]	14±2[c]
180	16±2	21±3	22±2	21±2	26±2[c]	37±3[be]
360	23±3	29±4	39±3	42±3[c]	37±3[c]	61±4[b]
1440	42±8	53±3	51±2	65±3[b]	69±4[b]	66±3[b]

(C)

	INTRACELLULAR DOXol (pmol) from DOX incubation					
TIME (min)	**H9c2 (heart)**	**PANC-1 (pancreas)**	**MCF-7 (breast)**	**A-498 (kidney)**	**HCT-15 (colon)**	**HepG2 (liver)**
20	4±1	5±1	5±1	9±2	7±2	9±1[c]
60	6±2	9±1	10±1	17±3[c]	14±2	16±2[cd]
180	13±4	17±3	18±3	26±3	21±2	29±3[c]
360	29±3	25±4	32±3	50±5[b]	45±3[b]	49±4[b]
1440	48±3	43±5	53±3	71±5[b]	67±4[b]	71±4[b]

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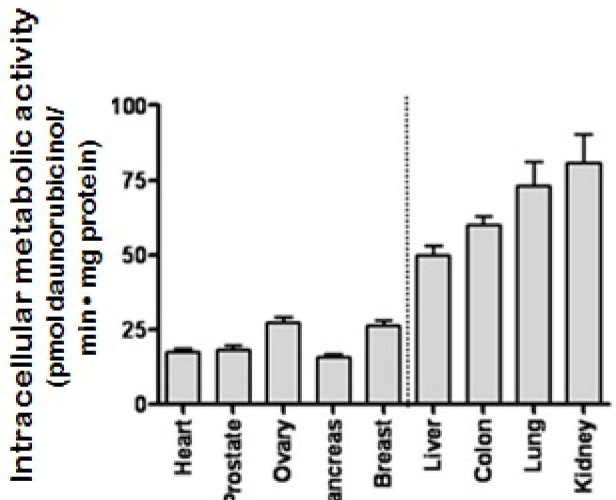
(D)

	EXTRACELLULAR DOXol (pmol) from DOX incubation					
TIME (min)	**H9c2 (heart)**	**PANC-1 (pancreas)**	**MCF-7 (breast)**	**A-498 (kidney)**	**HCT-15 (colon)**	**HepG2 (liver)**
20	18±3	21±2	26±2	54±4[b]	39±4[b]	60±3[b]
60	41±4	31±3	41±5	87±5[b]	59±3[b]	83±5[b]
180	62±5	56±7	62±6	162±10[b]	128±9[b]	149±9[b]
360	110±8	86±5	112±8	199±10[b]	178±11[b]	196±10[b]
1440	143±11	132±10	172±11	263±18[b]	228±16[b]	251±14[b]

The following symbols represent statistically significant differences ($p<0.05$): [a] denotes a value significantly different from all three drug-tolerant cell lines; [b] denotes a value significantly different from all three drug-sensitive cell lines; [c] denotes a value significantly different from the heart cell line; [d] denotes a value significantly different from the pancreas cell line; and [e] denotes a value significantly different from the kidney cell line.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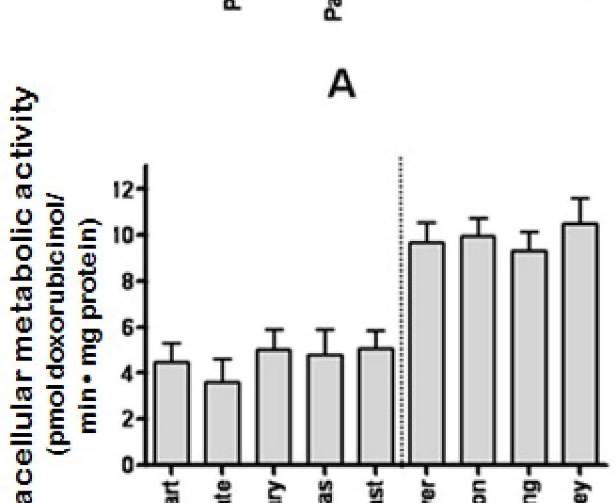
Heart
Prostate
Ovary
Pancreas
Breast
Liver
Colon
Lung
Kidney
A


Intracellular metabolic activity
(pmol doxorubicinol/
min • mg protein)
12
10
8
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4
2
0
Heart
Prostate
Ovary
Pancreas
Breast
Liver
Colon
Lung
Kidney
B

Figure 1

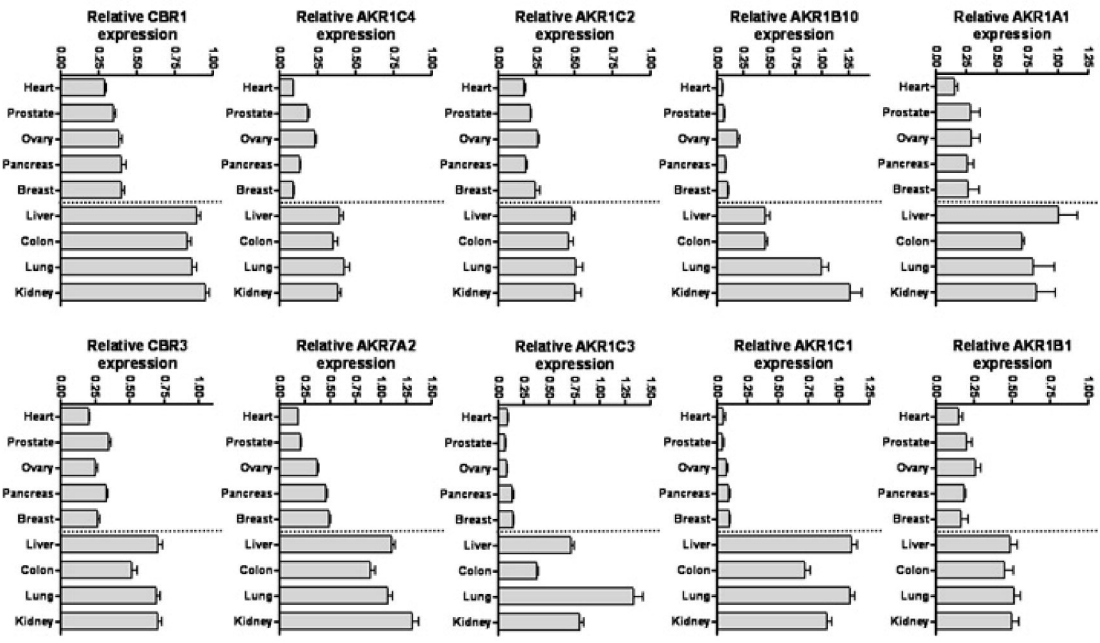
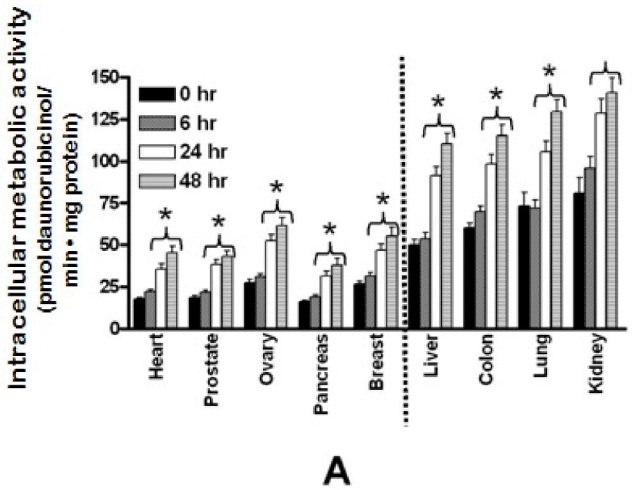

Relative CBR1 expression
Relative AKR1C4 expression
Relative AKR1C2 expression
Relative AKR1B10 expression
Relative AKR1A1 expression
Relative CBR3 expression
Relative AKR7A2 expression
Relative AKR1C3 expression
Relative AKR1C1 expression
Relative AKR1B1 expression
Heart
Prostate
Ovary
Pancreas
Breast
Liver
Colon
Lung
Kidney

Figure 2


Intracellular metabolic activity
(pmol daunorubicinol/
min • mg protein)
150
125
100
75
50
25
0
0 hr
6 hr
24 hr
48 hr
*
Heart
Prostate
Ovary
Pancreas
Breast
Liver
Colon
Lung
Kidney
A

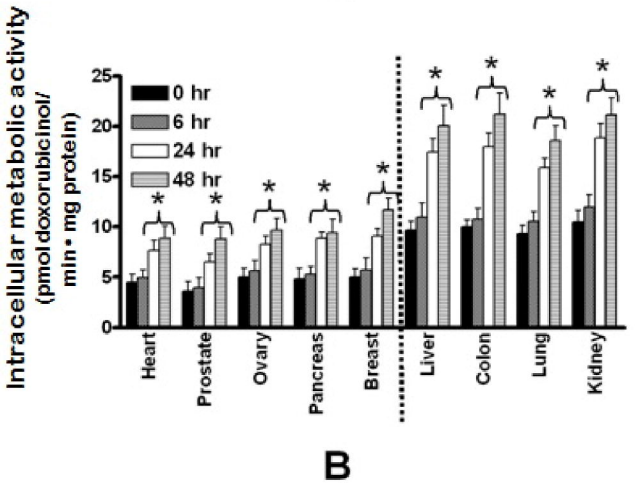
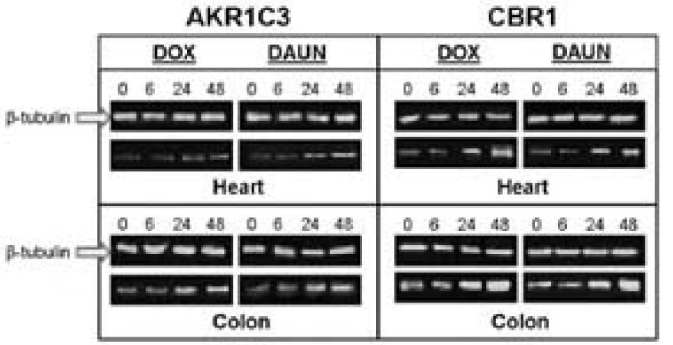
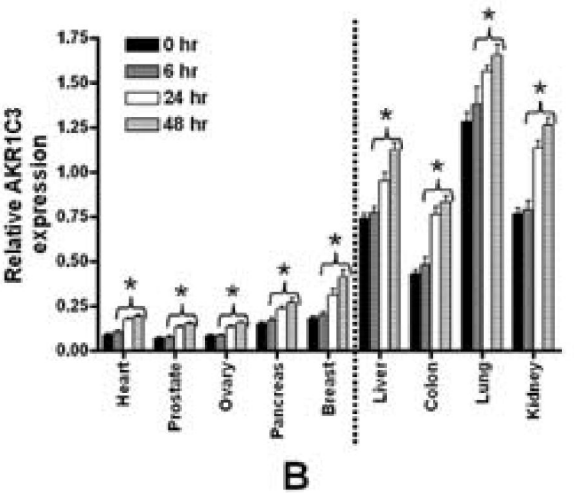

Intracellular metabolic activity
(pmol doxorubicinol/
min • mg protein)
25
20
15
10
5
0
0 hr
6 hr
24 hr
48 hr
*
Heart
Prostate
Ovary
Pancreas
Breast
Liver
Colon
Lung
Kidney
B

Figure 3



A



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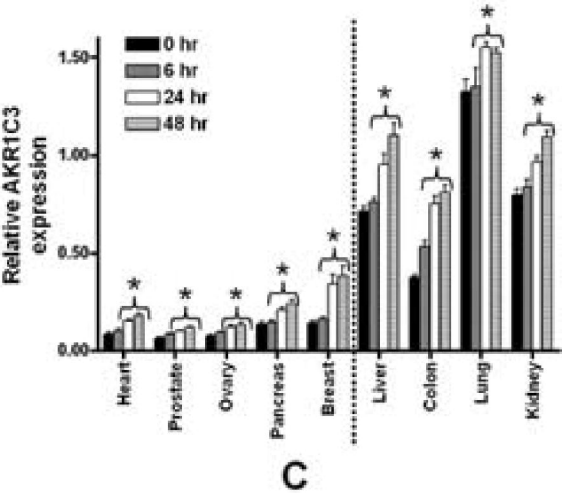

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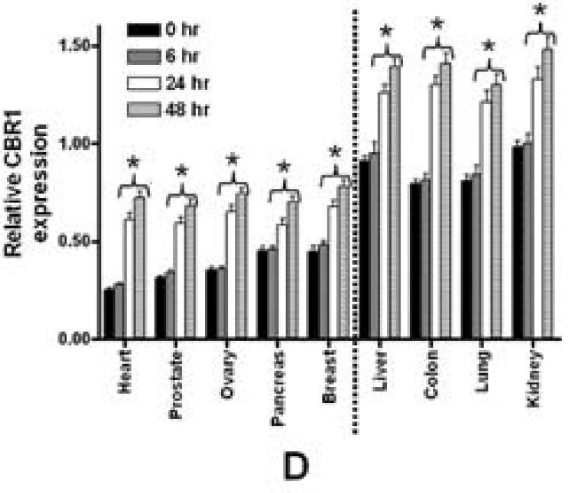

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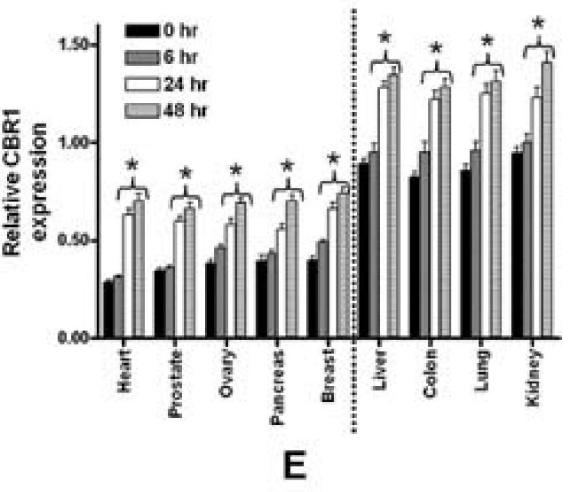

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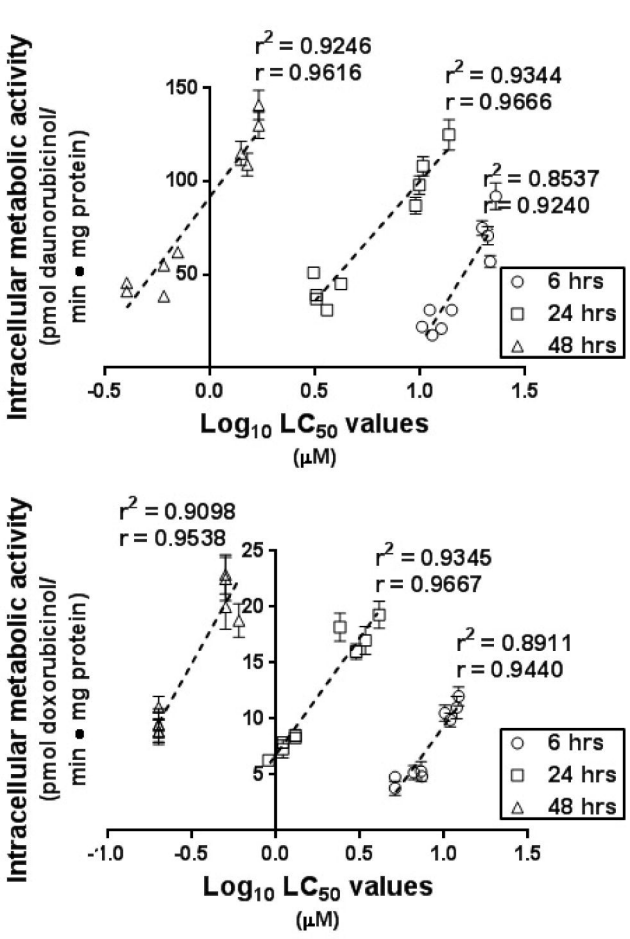
Figure 4


Intracellular metabolic activity
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min • mg protein)
r² = 0.9246
r = 0.9616
r² = 0.9344
r = 0.9666
r² = 0.8537
r = 0.9240
6 hrs
24 hrs
48 hrs
Log10 LC50 values
(μM)
Intracellular metabolic activity
(pmol doxorubicinol/
min • mg protein)
r² = 0.9098
r = 0.9538
r² = 0.9345
r = 0.9667
r² = 0.8911
r = 0.9440
6 hrs
24 hrs
48 hrs
Log10 LC50 values
(μM)

Figure 5