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Adipocytes Sequester and Metabolize the Chemotherapeutic Daunorubicin

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Abstract

Obesity is associated with poorer outcome for many cancers. Previously, we observed that adipocytes protect acute lymphoblastic leukemia (ALL) cells from the anthracycline, daunorubicin (DNR). In the present study, it is determined whether adipocytes clear DNR from the tumor microenvironment (TME).

Intracellular DNR concentrations were evaluated using fluorescence. DNR and its largely inactive metabolite, daunorubicinol, were analytically measured in media, cells, and tissues using liquid chromatography/mass spectrometry (LC/MS). Expression of DNR-metabolizing enzymes: aldo-keto reductases (AKR1A1, AKR1B1, AKR1C1, AKR1C2, AKR1C3, and AKR7A2) and carbonyl reductases (CBR1, CBR3) in human adipose tissue were queried using public databases, and directly measured by quantitative PCR (qPCR) and immunoblot. Adipose tissue AKR activity was measured by colorimetric assay.

Adipocytes absorbed and efficiently metabolized DNR to daunorubicinol reducing its anti-leukemia effect in the local microenvironment. Murine studies confirmed adipose tissue conversion of DNR to daunorubicinol in vivo. Adipocytes expressed high levels of AKR and CBR isoenzymes that deactivate anthracyclines. Indeed, adipocyte protein levels of AKR1C1, AKR1C2, and AKR1C3 are higher than all other human non-cancerous cell types. To our knowledge, this is

the first demonstration that adipocytes metabolize and inactivate a therapeutic drug. Adipocyte-mediated DNR metabolism reduces active drug concentration in the TME. These results could be clinically important for adipocyte-rich cancer microenvironments such as omentum, breast, and marrow. Since AKR and CBR enzymes metabolize several drugs, and can be expressed at higher levels in obese individuals, this proof-of-principle finding has important implications across many diseases.

Keywords

adipocytes; aldo-keto reductase; anthracyclines; microenvironment; leukemia

Introduction

It is well established that obesity increases the risk for cancer mortality (1). While obesity has been linked with poorer outcome from several cancers, including that of the breast (2), colon (3), ovary (4), and prostate (5), no mechanisms have been proven to explain these effects. One potential contributor to poor cancer outcome in obesity could be inadequacy of chemotherapy dosing. Excess adiposity can lead to alterations in chemotherapy pharmacokinetics (PK). Lipophilic chemotherapies can preferentially accumulate in adipose tissue (6), thus increasing the volume of distribution, and reducing cancer cells exposure to the chemotherapy. Practices of dose capping, dosing by body surface area, and adjusting for ideal or lean body weight could further contribute to underdosing in obese patients. However, few studies have systematically evaluated how obesity alters the disposition of chemotherapies in patients.

Anthracyclines such as daunorubicin (DNR) and doxorubicin (DOX) are important chemotherapy agents used in a wide variety of cancers in children and adults. We have shown that adipocytes protect acute lymphoblastic leukemia (ALL) cells from a variety of chemotherapies, including DNR (7,8). We report herein that adipocytes sequester DNR and metabolize it to an inactive form, showing for the first time that adipose tissue is a drug-metabolizing organ.

Materials and Methods

Materials

DNR and DOX were purchased from Sigma Chemicals (St. Louis, MO). Daunorubicinol and doxorubicinol were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Media and supplements were obtained from Gibco, Thermo Fisher Scientific (Waltham, MA). Fetal bovine serum (FBS) was purchased from Denville Scientific (Metuchen, NJ).

Tissue culture

Human ALL cell lines BV173 (Ph+ Pre B ALL) and SEM (t4;11 Pre B ALL) were acquired from American Type Culture Collection (ATCC), authenticated by short tandem repeats by the University of Arizona Genetics core on 11/2016, and tested negative for mycoplasma. ALL cell lines were cultured in RPMI 1640 containing 10% FBS, 1% sodium pyruvate, 1%

GlutaMax, and 0.1% gentamicin ("complete media"), and maintained at densities between 0.5–2.0×106 cells per mL in a humidified incubator with 5% CO2. The murine pre-adipocyte cell line 3T3-L1 from ATCC was differentiated into adipocytes as previously described (9) and used for experiments between days +7 and +11 of differentiation. As a control, undifferentiated 3T3-L1 cells were irradiated with 90 Gy to induce senescence and plated at confluence, referred to herein as 3T3-L1 fibroblasts. Immortalized human adipocytes (ChubS7) were also differentiated and cultured as previously described. Human bone marrow derived mesenchymal stem cells (MSC) were obtained from Thermo-Fisher and differentiated per manufacturer instructions with MesenPRO medium. In co-culture experiments, ~200,000 ALL cells were cultured in 0.4-μm pore-size polycarbonate TransWell (Corning, Inc.) over ~100,000 fibroblasts or adipocytes, or no feeder layer in 24-well plates. ALL cells and adipocytes were cultured with DNR for various time intervals. ALL cell viability experiments were done in 96 well plates, using 75–100,000 initial cells. In some experiments, BV173 cells were pre-loaded with DNR for 1 hour at 37°C, pelleted and re-suspended in ice cold PBS, and plated on TransWells over either no feeder or adipocyte, and then collected at designated time points over the next 4 hours for flow cytometry.

Experimental animals

All mouse experiments were approved by the CHLA IACUC and performed in accordance with the USPHS Policy on Humane Care and Use of Laboratory Animals. C57BL/6J diet-induced obese and control mice (raised respectively on 60 kCal% or 10 kCal% fat diet from Research Diets, New Brunswick, NJ) were purchased from The Jackson Laboratory (Bar Harbor, ME). Obese and control male mice were used as a source of adipose tissue explants at 4–6 months of age. Mice were anesthetized with ketamine and xylazine, and intra-cardiac perfusion performed with PBS until liver clearing prior to harvesting of tissues. Adipose tissue was rinsed with cold PBS, cut into ~100 mg pieces, and washed twice with RPMI plus 10% FBS prior to culture in media with DNR, For *in vivo* PK distribution, 3 obese and 3 control 13-week-old mice were injected with 5 mg/kg DNR via tail vein. Mice were anesthetized 2 hours after DNR injection as above, and blood samples collected via intra-cardiac puncture, followed by intra-cardiac perfusion as above. Blood, spleen, bone marrow, subcutaneous fat and omental fat were collected for DNR and daunorubicinol measurements using LC/MS (see below). Plasma was separated by centrifugation and white blood cells were collected using Ficoll-Paque (GE Healthcare Life Sciences) according to manufacturer's protocol. Results from control and obese mice were combined as they were not qualitatively or statistically different.

Human samples

All human samples were obtained and used after IRB approval and written informed consent and in accord with assurances filed with and approved by the US DHHS. Subcutaneous abdominal adipose tissue biopsies were collected from a subset of obese adult female postmenopausal breast cancer survivors enrolled in an exercise intervention study 0–24 weeks out from completing chemotherapy and/or radiation (reference (10); approved by the University of Southern California IRB; ClinicalTrials.gov: NCT01140282). Subjects were randomized to a supervised combined aerobic and resistance exercise program over 16

weeks or usual care, and underwent biopsy at baseline and after the intervention. Biopsies were collected as previously described (11). Adipose tissue biopsy samples were rinsed in normal saline, and then transported in saline on ice to CHLA (~30 minutes), where they were immediately cut into ~100 mg sections. Some of these were cultured in complete media for 24 hours, and then fresh media was added with DNR for experiments. While explant weight was closely matched between experiments, adipocyte number and viability were not assessed on fresh mouse or human tissues.

Bone marrow biopsy specimens were obtained from children aged 10–21 diagnosed with high-risk ALL, as previously described (12), under approval of the CHLA IRB (ClinicalTrials.gov: NCT01317940). Biopsies from day #29 (post-induction) were examined.

Flow cytometry

FACS analysis was done using a FACScan (BD Bioscience). Intracellular DNR was measured using the phycoerythrin (PE) channel, taking advantage of the natural fluorescence of DNR. MDR-1 surface expression was quantified using an APC-conjugated anti-human MDR-1 antibody from BioLegend (San Diego, CA) according to manufacturer's instructions. Cells stained with an APC isotype control antibody were used as a negative control. DAPI was used to distinguish live cells. For all samples, 1 to 5×10^4 events were collected.

Microscopy

Adipocytes were grown and differentiated on poly-D lysine coated coverslips for analysis. These coverslips could then be placed in the bottom chambers of the TransWells co-cultures. DNR fluorescence images were acquired with an LSM 700 confocal system mounted on an AxioObserver.Z1 microscope equipped with a $63 \times /1.4$ Plan-APOCHROMAT objective lens and controlled with ZEN 2009 software (Carl Zeiss Microscopy, Thornwood, NY). A 488 nm laser with a 560 nm long-pass filter was used for fluorescence excitation and emission. Transmitted laser light was collected to form a DIC image simultaneously with the fluorescence image.

Paraformaldehyde-fixed bone marrow samples were embedded with paraffin. Samples were sectioned, mounted, and subjected to antigen retrieval with Citrate Buffer, pH 6.0, overnight. Endogenous peroxidases were inactivated with 0.3% H_2O_2. Nonspecific staining was blocked with 10% normal goat serum and 1% BSA before staining with polyclonal rabbit anti-human AKR1C1 (GeneTex), AKR1C2 (Cell Signaling) or AKR1C3 (Origene), and detected with polymerized peroxidase labeled goat anti-rabbit immunoglobulin (Invitrogen; mouse adsorbed). The reaction was detected with DAB (Millipore) and counterstained with Harris hematoxylin (Sigma). Images were acquired on a Leica DMI6000B Inverted Microscope ($\times 40/1.25$) with a Color CCD Digital Camera.

qPCR analysis

Adipose tissue was flash frozen and then lysed with QIAzol (Qiagen) using TissueLyzer II according to the manufacturer's protocol (Qiagen). qPCR was performed as previously

described (Sheng X, et al., Int J Obes (Lond) 2014;38(2):315–20), with the following thermal profile: 10 minutes at 95°C followed by 40 repeats of 95°C for 15 seconds, 60 degrees for 1 minute, and a final dissociation stage of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. See Supplementary Table 1 for primer sequences.

Western blots

Human liver tissue lysate was from Abcam (ab29889). Total protein was extracted from adipocytes using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet I, 1 mM PMSF, 1% Halt Protease Inhibitor Cocktail (Thermo Scientific) and Phosphatase Inhibitor Cocktail Set II (Calbiochem)). Adipose tissue was ground with lysis buffer using an electric rotator with glass pestle, followed by 20 strokes in a Dounce homogenizer. Lysates were sonicated with Bioruptor® (Diagenode) for 10 min on ice and centrifuged for 15 minutes at 13,000g at 4° C. The supernatant was retained and protein concentration was quantified by BCA assay (Pierce Biotechnology). Proteins were separated using SDS-PAGE and transferred onto a nitrocellulose membrane using the iBlot 2 Dry Blotting System (Life Technologies). Membranes were blocked and then probed with specific primary antibodies, followed by HRP-linked secondary antibodies. Bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and luminescence recorded with ImageQuant LAS 4000 (GE Healthcare Life Sciences, Piscataway, NJ).

Liquid chromatography/mass spectrometry

Doxorubicin (DOX; 50 μL at 1 μg/mL) was added as an internal standard (DNR was the internal standard when DOX and doxorubicinol were being analyzed). The entire sample was disrupted, and protein precipitated using 900 μL of ice-cold methanol and centrifuged at 13,000 rpm at 4°C for 5 minutes. The supernatant was isolated and evaporated to dryness. Cellular residues were reconstituted with 50 μL methanol with 0.1% formic acid, and 25 μL was injected into a Shimadzu Prominence HPLC linked to a Sciex API 3000. Each of the analytes, DNR, daunorubicinol, and doxorubicin was quantified using specific MRM: $528.50 \rightarrow 363.3$, $530.60 \rightarrow 321.30$, $544.50 \rightarrow 361.1$, and $546.5 \rightarrow 363.2$ and 399.4, for DNR, daunorubicinol, DOX, and doxorubicinol, respectively.

Aldo-keto reductase activity assay

Activity was measured using a colorimetric assay based on (13). Briefly, a reaction mixture containing 20 uM of 9,10-Phenanthrenequinone (Sigma) and 200 uM βNADPH (Sigma) was added to 1 ug of purified enzyme (rhAKR1C3 from R&D Systems, rhCBR1 from MyBiosource, San Diego, CA) or 5 ug lysates (mouse 3T3-L1 adipocytes, human breast adipose tissue) in sodium phosphate buffer (0.1M, pH 6.0) in a final volume of 100 ul/well. Stoichiometry of the reactions was determined by monitoring the decrease in NADPH/H+ absorbance at 340 nm for up to 30 min. Specific activity (pmol/min/ug) was determined using blank-adjusted OD/min slope. In some experiments, indomethacin or luteolin (both 100 uM final concentration) were added to the enzymes or lysates 5 min prior to adding the reaction mixture.

Search of Public Databases

To determine whether human adipose tissue expresses enzymes known to metabolize anthracyclines, we evaluated four publicly available gene expression profile datasets (14–17), which included Affymetrix analyses of human subcutaneous and visceral adipose tissue from children and adults. Ranks for AKR and CBR genes were for analyzed each sample, independent of tissue source or clinical subgroup. When more than one detection probe was assigned to a gene, the one with the highest ranks were recorded. To investigate whether human adipose tissue expresses these enzymes at the protein level, we searched ProteomicsDB (18), a publically available, mass-spectrometry-based database of the human proteome. Log_{10} normalized protein expression of AKR/CBR enzymes in tissues was used to generate a heat map using Microsoft Excel.

Statistical Analysis

Data are shown as mean±SD. All experiments were performed at least three times. For flow cytometry of intracellular DNR and surface MDR-1 expression, median fluorescence intensity (MFI) was reported. Two-sided, paired Student's t tests were used to compare differences between the experimental conditions. A p-value of less than 0.05 was considered statistically significant.

Results

Adipocytes reduce DNR concentration in nearby ALL cells

We previously showed that adipocytes protect murine leukemia cells from DNR in both direct contact (7), and when separated by semi-permeable membranes (8). To determine whether this ALL drug resistance is a consequence of decreased concentration DNR in leukemic cells, we cultured human ALL cells for 24–48 hours in Transwells over no feeder or ~100,000 adipocytes. The presence of adipocytes significantly reduced DNR accumulation in ALL cells, measured by fluorescence (Fig. 1A). To control for total number of cells in each well, additional Transwells with ALL cells over ~100,000 3T3-L1 fibroblasts were used, and showed that fibroblasts did not affect ALL cell DNR concentration. The addition of adipocytes did not alter the surface expression of MDR-1 found on ALL cells, suggesting that reduced intracellular DNR in ALL was not the consequence of increased cellular efflux (Fig. 1B). To confirm this, BV173 cells were pre-loaded with 18 μM DNR for 1 hour and plated alone or over adipocytes; the presence of adipocytes did not alter DNR efflux from ALL cells (Fig. 1C, D).

Adipocytes absorb anthracyclines

To test whether adipocytes sequester DNR from the media, 3T3-L1 adipocytes were cultured with DNR for 4 hours. Adipocytes accumulated DNR (measured by fluorescence) in a concentration dependent manner, with signal visible primarily in the cytoplasm (Fig. 2A). To investigate whether this adipocyte sequestration would reduce media DNR cytotoxicity, we pre-incubated media with 100 nM DNR (the EC_{90} dose for BV173) with no feeder cells, fibroblasts or adipocytes. ALL cells survived and proliferated better in media that had been pre-incubated with adipocytes when compared to fibroblasts or no feeder cells (Fig. 2B).

This ability of adipocytes to detoxify the media was detectable even when DNR concentrations as high as 1000 nM, albeit with diminished efficacy (Fig. 2C). Lysates from adipocytes cultured in high DNR concentrations were toxic to BV173 cells (Fig. 2D), confirming that adipocytes did indeed sequester DNR from the media in sufficient quantities to allow ALL cells to resist the DNR cytotoxicity. DNR did not induce any significant morphological changes or cell death in adipocytes by microscopy (not shown).

To determine whether adipocytes sequestered other anthracyclines and related drugs, we incubated 3T3-L1 adipocytes with various concentrations of DOX and mitoxantrone for 16 hours. Adipocytes decreased the concentrations of both DOX (Fig. 2E) and mitoxantrone in the media, measured by LC/MS.

Adipocytes metabolize DNR

While adipocytes detoxified very high concentrations of DNR from the media, their lysates were not as toxic as one would expect based on accumulation alone. We therefore hypothesized that adipocytes were metabolizing DNR. Since fluorescence cannot differentiate DNR from its major metabolite, daunorubicinol, we used LC/MS to quantify both compounds. When cultured in DNR for 16 hours, BV173 ALL cells accumulated DNR, with nearly undetectable concentrations of daunorubicinol (Fig. 3A). However, when adipocytes were present, ALL cell DNR concentrations were only ~1/3rd as high.

Adipocytes took up DNR in culture, but interestingly accumulated higher concentrations of daunorubicinol, a less cytotoxic metabolite (Fig. 3B). This suggests that adipocytes can deactivate DNR. This finding was affirmed using human MSC-derived adipocytes, which also showed intracellular accumulation of DNR and daunorubicinol (not shown). Further, adipocytes rapidly reduced media DNR concentration (Fig. 3C), while increasing media daunorubicinol concentration (Fig. 3D). Both of these effects were greater than observed in undifferentiated 3T3-L1 fibroblasts.

DNR metabolism by adipose tissue

To test whether intact adipose tissue would sequester and metabolize DNR, we incubated mouse adipose tissue explants in DNR. After 16 hours, adipose tissue removed DNR from the media, replacing much of it with released daunorubicinol (Fig. 4A). Adipose tissue accumulated both the cytotoxic DNR and the inactivated daunorubicinol, demonstrating that intact adipose tissue can sequester, metabolize and inactivate DNR (Fig. 4B). This experiment was performed with adipose tissue from various anatomical sites, and demonstrated that adipose from all depots can efficiently metabolize DNR to daunorubicinol. Human subcutaneous adipose tissue biopsy specimens also accumulated and metabolized DNR, albeit with a high degree of variability in these experiments (Fig. 4C).

In vivo DNR distribution

Although a comprehensive pharmacokinetics (PK) experiment was beyond the scope of the present study, we tested whether adipose tissue would accumulate and metabolize DNR *in vivo*. Two hours after a DNR injection, plasma DNR and daunorubicinol reached similar concentrations (Fig. 4D). While DNR was detectable in the spleen, bone marrow, and

circulating WBC, little to no daunorubicinol was detected in these cells. In contrast, adipose tissue accumulated both DNR and daunorubicinol. The ratio of daunorubicinol to DNR was 0.60±0.26 and 0.55±0.21 in subcutaneous and omental adipose tissue, respectively. By contrast, this ratio was significantly lower in WBC (0.16±0.11, p<0.001 vs. both adipose tissues), and undetectable in spleen and marrow. These findings suggest that adipose tissue actively converts DNR to daunorubicinol *in vivo*.

Adipocytes express DNR metabolizing enzymes

There are a number of enzymes capable of convert DNR to daunorubicinol (19,20), and so we next evaluated whether human adipose tissue expresses these enzymes. Four publically available gene expression profiles (14–17) showed that human adipose tissue expressed high levels of many of these enzymes, including aldo-keto reductase (AKR)1A1, AKR1B1, AKR1C1, AKR1C2, AKR1C3, AKR7A2, carbonyl reductase (CBR)1, and CBR3 (Fig. 5A). AKR1B10 and AKR1C4 were not expressed (not shown). qPCR of human adipose tissue biopsy specimens confirmed high gene expression of all of these metabolic enzymes relative to β-actin (Fig. 5B), as well as undetectable levels of AKR1B10 and 1C4. ProteomicsDB, a publically available database of human cellular proteomics (18), showed that adipocytes express high protein levels of these enzymes (Fig. 5C). Of note, this database showed adipocytes to have the highest protein levels of AKR1C1, AKR1C2, and AKR1C3 of all non-cancerous tissues evaluated. Western blots confirmed protein expression of these AKR and CBR enzymes, with the exception of CBR3, in human subcutaneous adipose tissue biopsies and the human adipocyte cell line, ChubS7 (Fig 5D). Further, AKR1C1, 1C2, and 1C3 were also shown to be present in the cytoplasm of bone marrow adipocytes in children being treated for ALL (Fig 5E).

Adipose tissue aldo-keto reductase activity

To further verify that adipose tissue has AKR activity, we utilized a colorimetric assay based on NADPH (13). Lysates of both subcutaneous human adipose tissue biopsies and murine 3T3-L1 adipocytes showed AKR activity (Fig 6A). This AKR activity was inhibited by the AKR1C inhibitor, indomethacin, and the CBR1 inhibitor, luteolin, though the latter did not reach statistical significance (Fig 6B).

Discussion

In the current study, we present the novel finding that adipocytes sequester and metabolize the anthracycline, DNR. This is the first report, to our knowledge, showing that adipocytes can metabolize and inactivate a therapeutic agent. This metabolism of DNR, as well as sequestration of other chemotherapies such as DOX and mitoxantrone, could reduce the concentration of active drugs in adipocyte-rich microenvironments, such as adipose tissue, omentum, and bone marrow. This is of particular importance since during leukemia treatment, bone marrow exhibits substantial fat accumulation (21). Further, cancer treatment induces large increases in whole body adiposity (22), and obesity itself has been associated with higher adipose tissue expression of some of these enzymes (23,24). Together, these changes may contribute to local reduction of cytotoxic activity of chemotherapy, leading to emergence of drug resistant tumor cells and risk for treatment failure. We highlight a new

role of the adipocyte in the emergence of chemotherapy resistance in the tumor microenvironment.

Anthracyclines are broadly used in treatment regimens for a wide variety of cancers, including leukemia, lymphoma, ovarian, pancreatic, breast cancers, bone and soft tissue cancers. This new finding that fat can sequester and deactivate cytotoxic chemotherapy has wide implications and may partially explain why obese patient have poorer clinical response when compared to their leaner counterparts. Additionally, the AKR and CBR isoenzymes are highly expressed in adipocytes and are known to metabolize a wide range of drugs; thus, it is possible that adipocytes could impact the efficacies of other drugs in relevant microenvironments.

We noted that adipocytes were less efficient in metabolizing DOX when compared to DNR. This was an unexpected finding since these anthracyclines differ by only one hydroxyl group, and they are cleared by similar isoenzymes (19). Decreased DOX metabolism by adipocytes may reflect differing enzyme affinity between the two anthracyclines. For example, AKR1A1 metabolizes DNR but not DOX (25). Both DNR and DOX are substrates for AKR1B10, AKR1C1, AKR1C3, AKR7A2, and CBR1, but these enzymes preferentially metabolize DNR when compared to DOX (19,26). On the other hand, others have reported that AKR expression contributes to DOX resistance in breast cancer cells, even in the absence of detectible doxorubicinol accumulation in these cells or media (27). Nonetheless, adipocytes accumulated DNR, DOX, and mitoxantrone, suggesting that adipocytes can sequester all of these chemotherapies from their microenvironment, with differences only in subsequent intracellular metabolism.

While the rapid uptake and efficient deactivation of DNR by adipocytes reduces DNR concentration in the microenvironment and in nearby leukemia cells, the mass effect is insufficient to clearly alter the drug's plasma PK. Because the vast majority of anthracycline clearance from plasma occurs in the liver and kidney, peripheral adipocyte sequestration and metabolism would not be expected to significantly alter the plasma profile. This partially explains why Thompson et al. found that neither BMI nor body fat were significantly correlated with DOX or DNR plasma clearance in children (28,29). In fact, adiposity might be associated with a *decreased* plasma anthracycline clearance or *increased* AUC in adults (see for example (30)). However, treatment failures and relapses in leukemia are most often present in the bone marrow, where our results show that increased adiposity could reduce available anthracycline levels. As we have previously shown that ALL cells migrate into adipose tissue under the influence of the chemokine CXCL-12 (31), adipose tissue itself could be an unrecognized sanctuary site for leukemia cells, where anthracyclines are unable to reach therapeutic levels. Thus, adipocyte anthracycline sequestration and metabolism may contribute to survival of local leukemia clones within the bone marrow and adipocyte, thus increasing the risk of residual disease at the end of Induction therapy and eventual relapse.

In addition to reducing active DNR concentrations in the leukemia microenvironment, adipocyte-mediated DNR metabolism could contribute to its major long-term toxicity, cardiotoxicity. While much less cytotoxic to ALL cells than the parent compound, daunorubicinol has a longer half-life in both plasma and cardiac tissue, and has been shown

to disproportionately contribute to cardiac toxicity (32). Adipocyte sequestration and slow release of daunorubicinol could result in an increased plasma half-life of this metabolite. This line of thought is supported by a PK study showing that doxorubicinol plasma clearance was decreased in children with >30% body fat (28). Thus, this mechanism may contribute to the observed link between obesity and anthracycline-related cardiotoxicity observed in animal models (33) and childhood cancer survivors (34).

There are some limitations to be considered in the data presented. While we have shown that adipocytes metabolize DNR *in vivo*, we have not tested whether this leads to lower active DNR concentration in nearby cancer cells *in vivo*, nor whether obesity alters ALL cell DNR concentrations *in vivo*. In addition, although we showed that adipocytes take up DNR at a much higher rate than ALL cells and fibroblasts, we have not characterized these uptake kinetics. These are important future experiments that should be done to further characterize these effects and help confirm the clinical relevance of the present studies. Furthermore, we identified the presence of several anthracycline metabolizing enzymes in adipocytes, but have not identified the individual contribution that each enzyme has on DNR metabolism. This level of detail is important, given the large number of these enzymes and isoforms, and their roles in metabolizing hormones, medications, and toxins. Although we are not the first to show that adipocytes express some AKR and CBR enzymes (18), our findings that adipocytes express these enzymes at very high levels compared to other tissues (Figure 4) and that fat cells sequester and detoxify anthracyclines are highly novel. Adipose may be an underappreciated metabolic tissue that can influence cancer outcomes by creating a sanctuary microenvironment for ALL cells. Our use of cell culture, mouse experiments, and human tissues strengthen the veracity and clinical relevance of these findings.

In conclusion, this is the first report demonstrating that adipocytes sequester and efficiently metabolize a pharmaceutical agent. Specifically, adipocytes metabolize DNR to a less toxic metabolite, and allow nearby ALL cells to evade DNR-induced cytotoxicity. This finding could help explain why obese cancer patients are at risk of having a poorer outcome. PK studies specifically in the tumor microenvironment will be necessary to determine the precise impact of adiposity on anthracycline-based treatment and efficacy in patients with ALL and other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Implications

Adipocyte absorption and metabolism of chemotherapies can reduce cytotoxicity in cancer microenvironments, potentially contributing to poorer survival outcomes
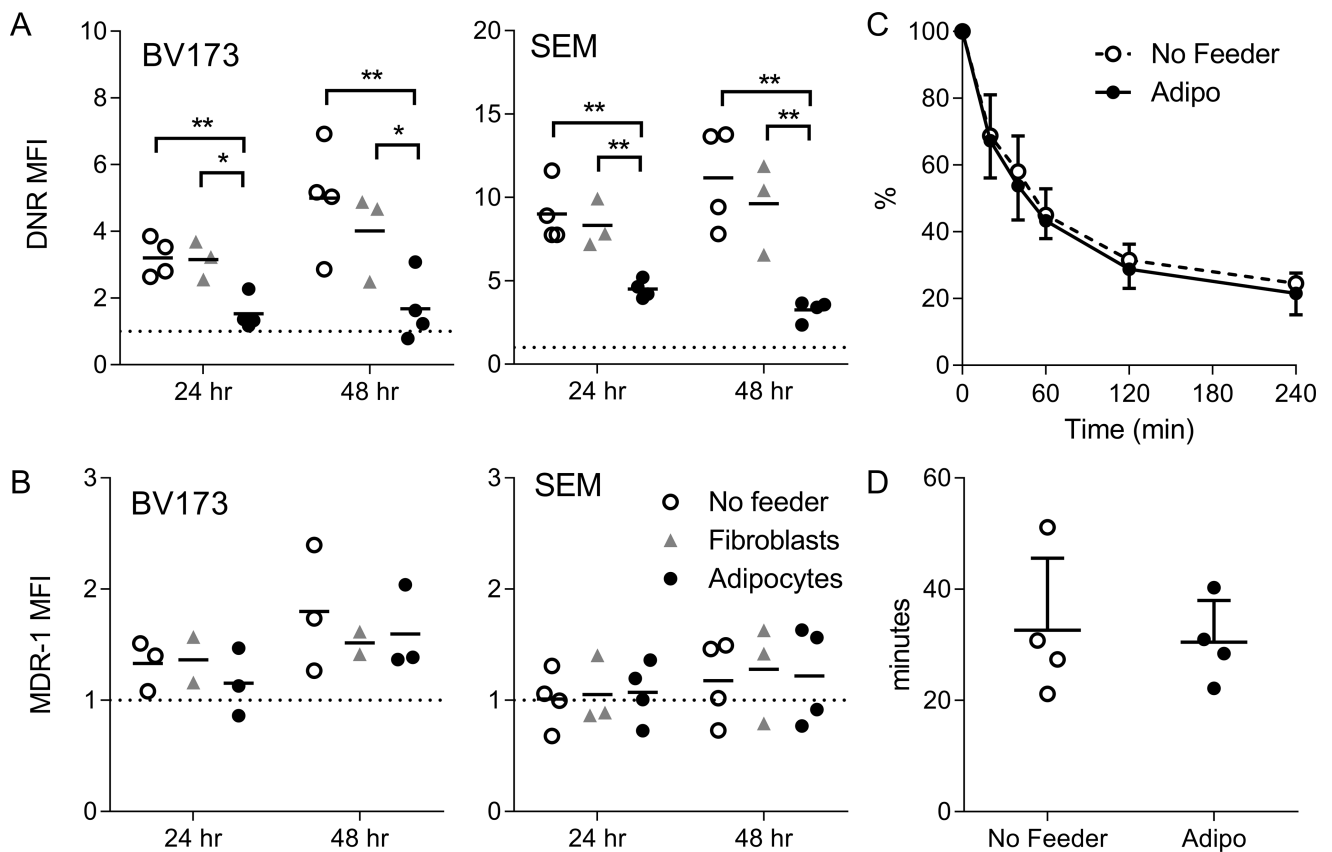
Figure 1. Adipocytes reduce DNR concentration in nearby ALL cells
A. DNR concentration by flow cytometry based on the natural fluorescence of DNR, in BV173 and SEM leukemia cells cultured with DNR in TransWells with no feeder (open circles), fibroblasts (gray triangles), or adipocytes (closed circles). Doses between 60 to 200 nM DNR were used to achieve 50 to 80% apoptosis after 48 hours of treatment. **B.** MDR-1 surface expression in ALL cells cultured with DNR over no feeder, fibroblasts, or adipocytes. **C.** BV173 pre-loaded with DNR were plated in TransWells over no feeder (open circles) or adipocytes (closed circles; n=4). Intracellular DNR was measured using flow cytometry at the indicated time points. There was no significant difference in efflux between the two conditions. **D.** Half-lives from curves shown in C (n=4). *p<0.05, **p<0.01.
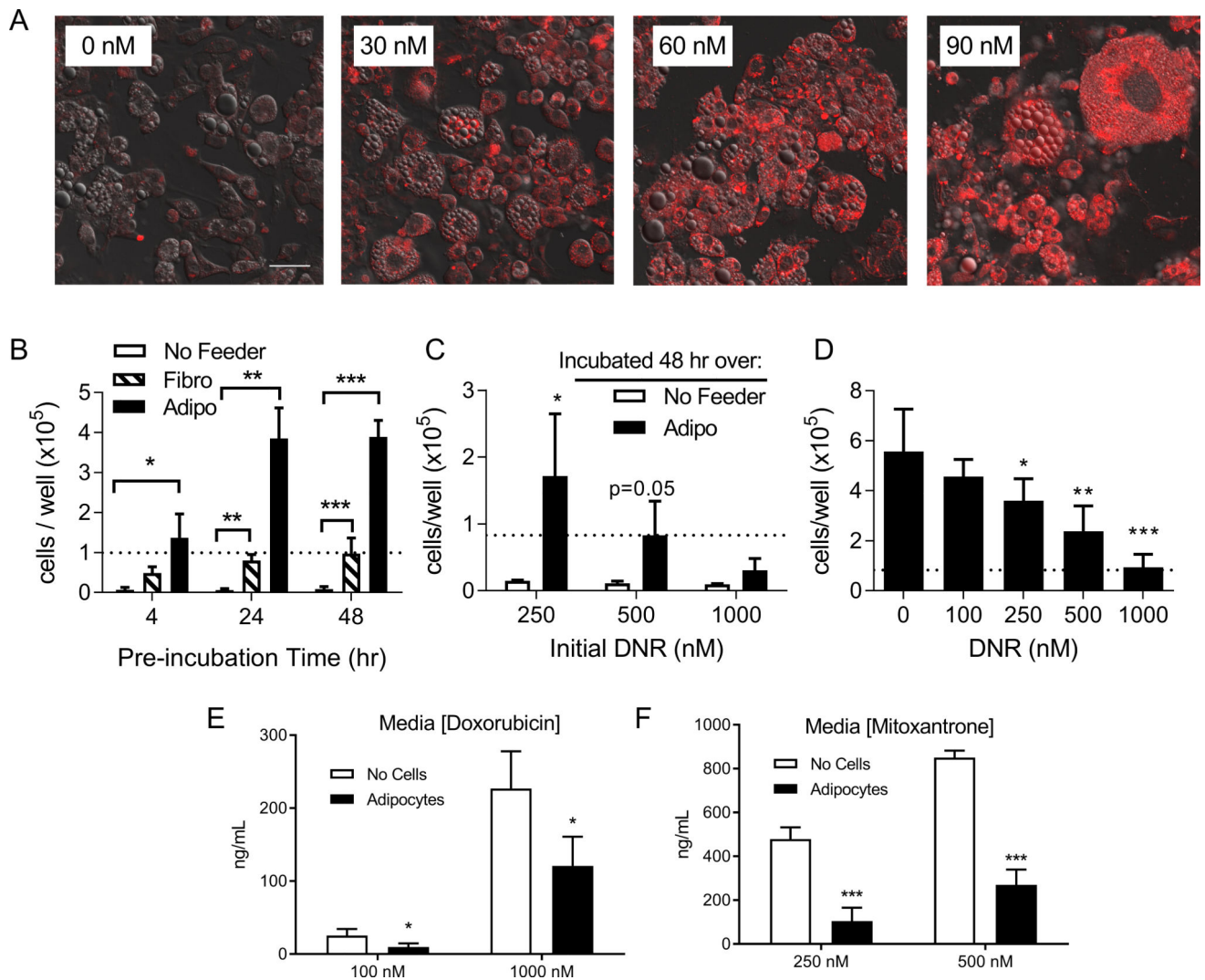
Figure 2. Adipocytes sequester DNR
A. Confocal microscopy pictures of adipocytes treated with different concentrations of DNR (red) for 4 hours. Scale bar denotes 50μm. **B.** Viable BV173 ALL cells cultured for 72 hours in media in which 100 nM DNR had been pre-incubated for the indicated time over no feeder (open), fibroblasts (hatched), or adipocytes (solid bars; dotted line indicates initial cell number; n=4). **C.** Viable BV173 ALL cells cultured for 72 hours in media which had been pre-incubated over no feeder (open) or adipocytes (solid bars) for 48 hours with various initial DNR concentrations. P values indicate comparisons to No Feeder conditions (n=3). **D.** Viable ALL cells after 72 hours in culture with lysates of adipocytes that had been preincubated with DNR for 48 hours. P values indicate comparisons to the 0 DNR lysate condition (n=3 for 100 nM, n=6 for all other doses). **E, F.** Media DOX (E) and mitoxantrone (F) concentrations after 16 hour incubation of each drug at the indicated concentrations alone (open) or over 3T3-L1 adipocytes (closed bars; n=4–6). *p<0.05, **p<0.01, and ***p<0.001
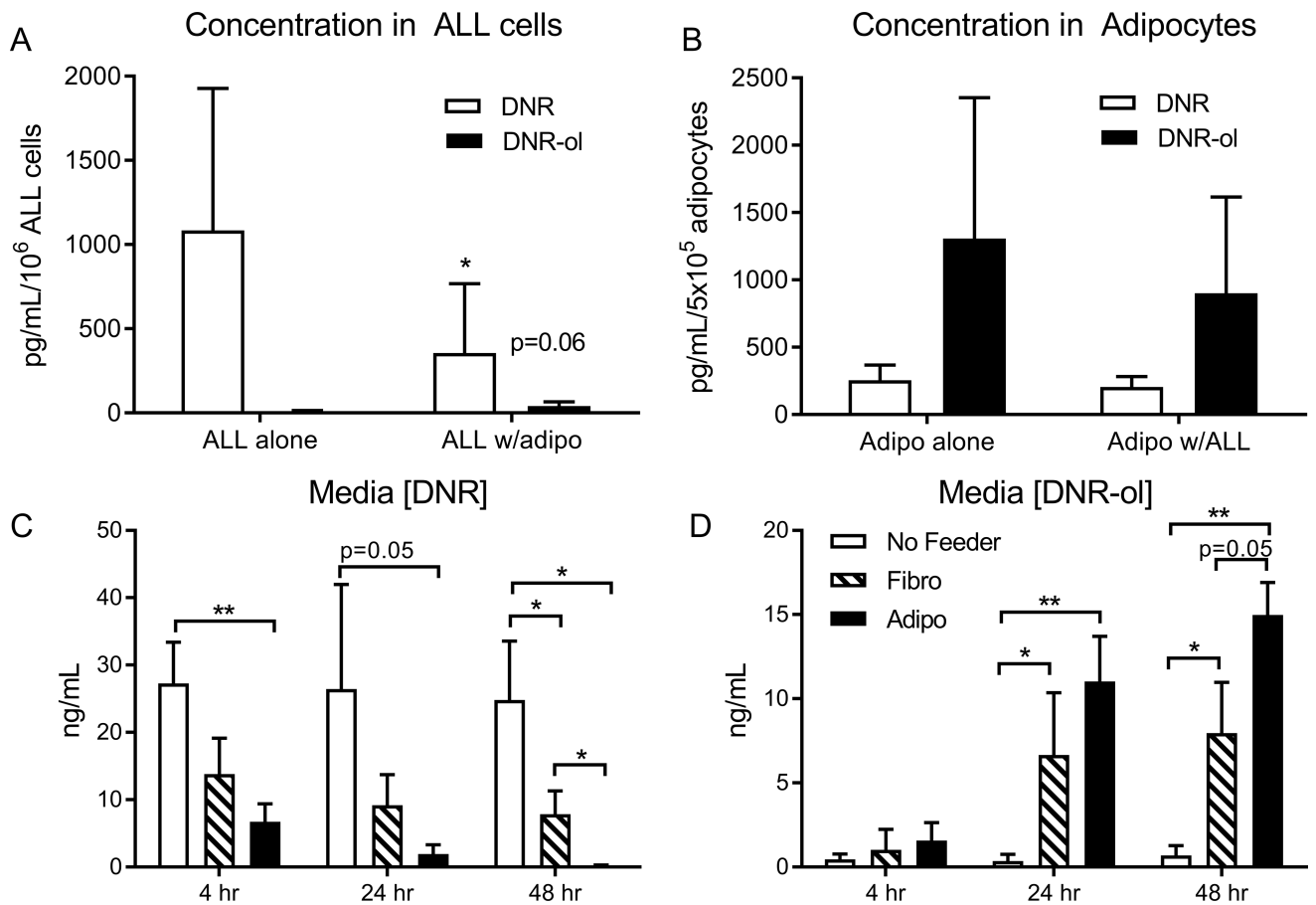
Figure 3. Adipocytes metabolize DNR into daunorubicinol
A. DNR and daunorubicinol concentrations in BV173 ALL cells cultured in 100 nM DNR alone or in transwells over adipocytes. P values indicate comparisons to ALL alone conditions. **B.** DNR and daunorubicinol concentrations in the adipocytes cocultured in graph A. **C,D.** Concentration of DNR (C) and daunorubicinol (D) in media cultured with no feeder cells, fibroblasts or adipocytes for various times (n=4). *p<0.05, **p<0.01, and ***p<0.001.
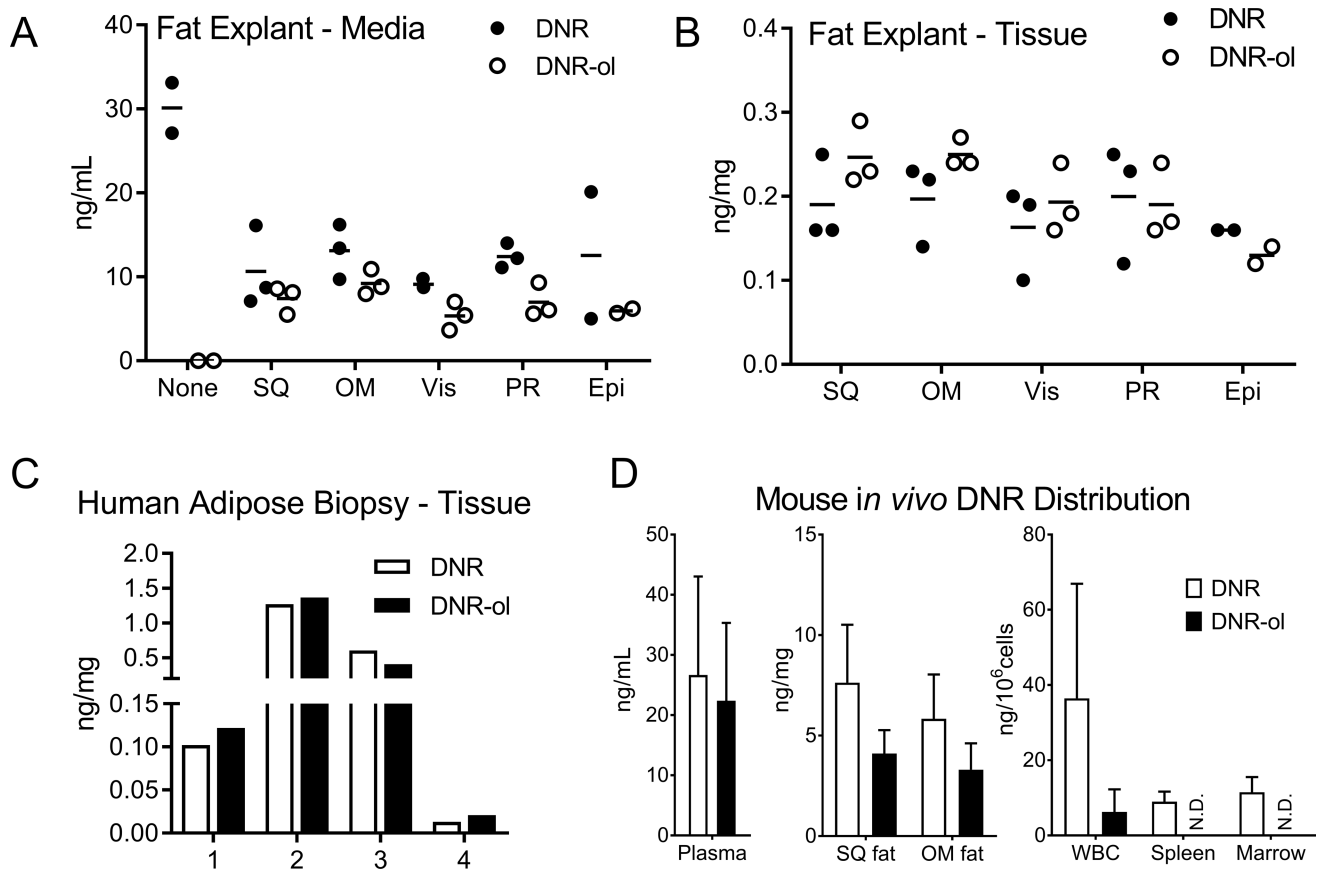
Figure 4. Intact adipose tissue metabolizes DNR

A, B. DNR (open) and daunorubicinol (closed bars) concentrations in media (A) and adipose tissue (B) after 100 mg portions of intact adipose explants from mice were incubated in DNR for 16 hours. **C.** DNR and daunorubicinol concentration in 100 mg portions of human adipose tissue biopsy material after incubation in 100 nM DNR for 16–24 hours. **D.** Intracellular and tissue DNR and daunorubicinol concentrations in various tissues taken 2 hours after intravenous injection of DNR at 5 mg/kg (n=6 mice). Daunorubicinol was undetectable in spleen and bone marrow cells (N.D.).
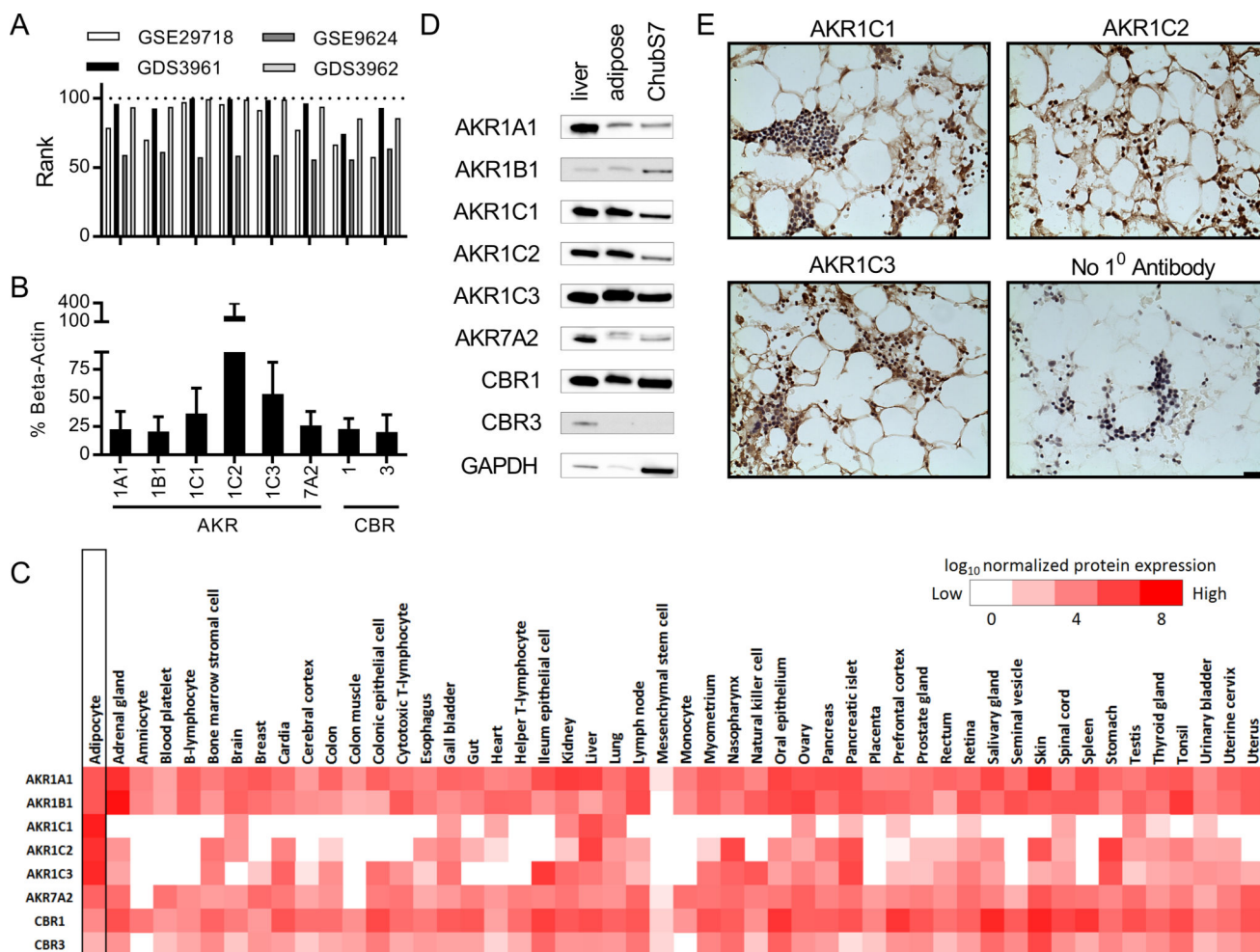
Figure 5. Expression of anthracycline metabolizing enzymes in human adipocytes
A. Gene expression ranks of selected AKR and CBR enzymes in human adipose tissue from four publically available gene expression profiles (mean rank without SD shown for clarity). **B.** Gene expression of AKR and CBR enzymes in human adipose tissue biopsy specimens (see text for subject details), by rtPCR, relative to β-actin (n=4). **C.** Protein expression of AKR and CBR enzymes in human tissues from ProteomicsDB (18). **D.** Western blots showing protein expression of AKR and CBR enzymes in human adipose tissue biopsy specimen (25 ug protein), immortalized human adipocytes (ChubS7, 25 ug protein), and liver (10 ug protein) for comparison. **E.** Immunohistochemistry of bone marrow biopsy specimens from one of three representative children with ALL at day #29, after Induction chemotherapy. HRP signal can be seen in adipocyte cytoplasm as a brown color. 40X; Bar = 25 um.
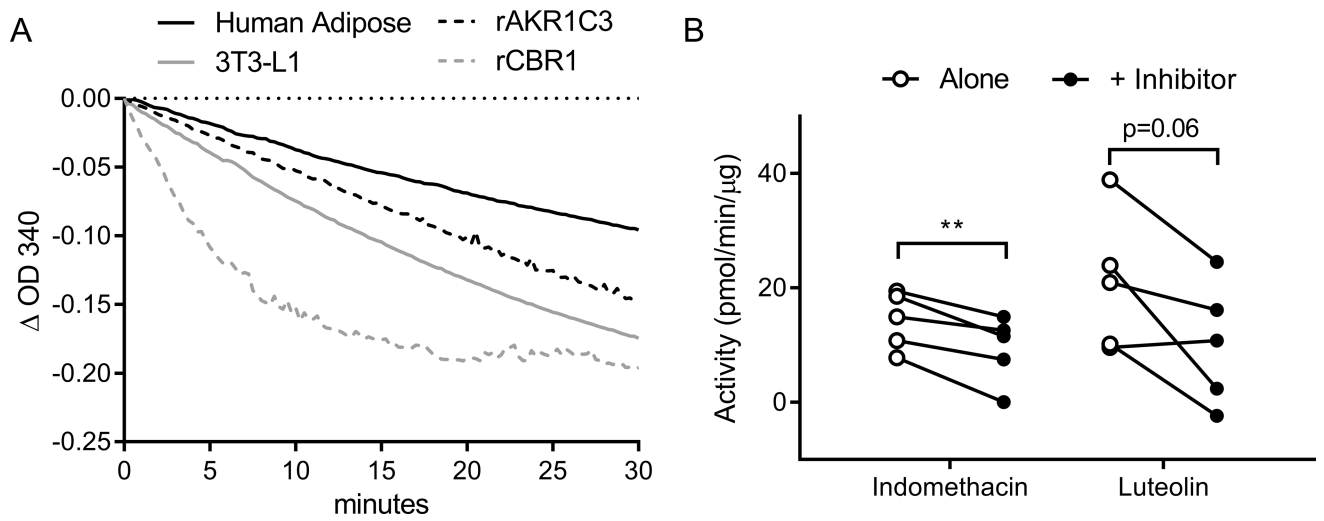
Figure 6. Adipocytes exhibit aldo-keto reductase activity
A. AKR activity measured by PQ reduction by colorimetric assay demonstrates activity in human adipose tissue (n=5 subjects, each measured on three separate occasions), murine 3T3-L1 cells (n=7), and recombinant enzymes (n=3–4). **B.** Adipose tissue aldo-keto reductase activity in the presence of two putative AKR inhibitors, indomethacin and luteolin, measured by PQ reduction (n=5). **p<0.01, two-sided paired t-test.