Published in final edited form as:

Toxicol Appl Pharmacol. 2011 August 15; 255(1): 40–47. doi:10.1016/j.taap.2011.05.014.

AKR1B10 Induces Cell Resistance to Daunorubicin and Idarubicin by Reducing C13 Ketonic Group

Linlin Zhong^{1,¶}, Honglin Shen^{2,¶}, Chenfei Huang¹, Hongwu Jing³, and Deliang Cao^{1,*}

¹ Department of Medical Microbiology, Immunology, & Cell Biology, Simmons Cancer Institute, Southern Illinois University School of Medicine. 913 N. Rutledge Street, Springfield, IL 62794-9626

² Department of Pediatrics, the Affiliated Shenzhen Nanshan Hospital of Guangdong Medical College. 89 Taoyuan Road, Nanshan District, Shenzhen 518052, China

³ 670 Wiese Road, Cheshire, CT 06410

Abstract

Daunorubicin, idarubicin, doxorubicin and epirubicin are anthracyclines widely used for the treatment of lymphoma, leukemia, and breast, lung, and liver cancers, but tumor resistance limits their clinical success. Aldo-keto reductase family 1 B10 (AKR1B10) is an NADPH-dependent enzyme overexpressed in liver and lung carcinomas. This study was aimed to determine the role of AKR1B10 in tumor resistance to anthracyclines. AKR1B10 activity toward anthracyclines was measured using recombinant protein. Cell resistance to anthracycline was determined by ectopic expression of AKR1B10 or inhibition by epalrestat. Results showed that AKR1B10 reduces C13-ketonic group on side chain of daunorubicin and idarubicin to hydroxyl forms. *In vitro*, AKR1B10 converted daunorubicin to daunorubicinol at V_{max} of 837.42 ± 81.39 nmol/mg/min, K_m of 9.317 ± 2.25 mM and k_{cat}/K_m of 3.24. AKR1B10 showed better catalytic efficiency toward idarubicin with V_{max} at 460.23 ± 28.12 nmol/mg/min, K_m at 0.461 ± 0.09 mM and k_{cat}/K_m at 35.94. AKR1B10 was less active toward doxorubicin and epirubicin with a C14-hydroxyl group. In living cells, AKR1B10 efficiently catalyzed reduction of daunorubicin (50nM) and idarubicin (30nM) to corresponding alcohols. Within 24 hours, approximately $20 \pm 2.7\%$ of daunorubicin (1 μ M) or $23 \pm 2.3\%$ of idarubicin (1 μ M) was converted to daunorubicinol or idarubicinol in AKR1B10 expression cells compared to $7 \pm 0.9\%$ and $5 \pm 1.5\%$ in vector control. AKR1B10 expression led to cell resistance to daunorubicin and idarubicin, but inhibitor epalrestat showed a synergistic role with these agents. Together our data suggests that AKR1B10 participates in cellular metabolism of daunorubicin and idarubicin, resulting in drug resistance. These data are informative for the clinical use of idarubicin and daunorubicin.

Keywords

Aldo-keto reductase family 1 member B10; anthracyclines; drug resistance; enzyme kinetics; drug metabolism

© 2011 Elsevier Inc. All rights reserved.

Correspondent: Deliang Cao, Department of Medical Microbiology, Immunology & Cell Biology, and Simmons Cancer Institute, Southern Illinois University School of Medicine. 913 N. Rutledge Street, Springfield, IL 62794-9626. Tel.: 217-545-9703; Fax: 217-545-9718; dcao@siu.edu.

[¶]Authors contributed equally.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Anthracyclines are a class of antitumor agents widely used for the treatment of leukemia, lymphoma, and breast, ovarian, liver and lung cancers (Wiernik and Dutcher, 1992; Murphy *et al.*, 1995; Moretti *et al.*, 2009). Daunorubicin is the first anthracycline developed (Dimarco *et al.*, 1964). Thereafter, more than 2000 derivatives have been developed, among which idarubicin, doxorubicin, and epirubicin are the most effective agents used in anticancer therapy (Muggia and Green, 1991; Moretti *et al.*, 2009). Anthracyclines efficiently arrest cell division and induce apoptotic cell death by intercalation with DNA, generation of free radicals, interaction with cellular membranes, and inhibition of topoisomerase II (Gewirtz, 1999; Hussein, 2007). However, drug resistance and side effects such as cardiotoxicity seriously limit their clinical success (Shan *et al.*, 1996; Den Boer *et al.*, 1998), and it is critical to understand their intracellular metabolism and pharmacokinetics.

Cancer cell resistance toward anthracyclines, intrinsic or acquired, is induced by multiple factors, such as multidrug resistant protein expression, apoptotic pathway alterations, and drug-detoxifying enzyme induction (Gianni, 1997; Den Boer *et al.*, 1998; Gottesman, 2002; Hembruff *et al.*, 2008). Intracellularly, anthracyclines are metabolized by either glycosidic cleavage of bio-functional glycosidic amino sugar or reduction of ketonic groups. Glycosidic cleavage of anthracyclines produces a biologically inactive 7-deoxyanthracyclinone (7-deoxyaglycone) (Asbell *et al.*, 1972; Niitsu *et al.*, 2000), whereas the reduction of C13 ketonic group leads to an alcoholic metabolite, such as daunorubicinol that has less antitumor activity but stronger cardiotoxicity (Beran *et al.*, 1979; Kuffel *et al.*, 1992). Carbonyl reductases (CBR), aldose reductase (AR), aldehyde reductase (ALR1), dihydrodiol dehydrogenase (DD), and cytochrome P450 are NAD(P)H-dependent enzymes that can reduce anthracyclines to corresponding alcoholic forms (Felsted and Bachur, 1982; Wermuth *et al.*, 1988; Ohara *et al.*, 1995; Lee *et al.*, 2001; Petrash, 2004; Schroterova *et al.*, 2004). It has been reported that in daunorubicin-resistant cells, CBR, ALR1 and DD are overexpressed. In these cells, glutathione S-transferases and multidrug resistance-associated proteins were all excluded as factors for daunorubicin resistance and therefore, the daunorubicin resistance is virtually ascribed to the induction of these reductases and their enzymatic detoxification (Ohara *et al.*, 1995; Plebuch *et al.*, 2007; Gavelova *et al.*, 2008). Interestingly, reductases AR, ALR1, and DD are aldo-keto reductase family 1 (AKR1) proteins, where aldo-keto reductase family 1 member B10 (AKR1B10) belongs (Chung and LaMendola, 1989; Jez *et al.*, 1997; Hyndman *et al.*, 2003; Liu *et al.*, 2009).

AKR1B10 is a novel NADPH-dependent AKR protein primarily expressed in the human intestine and adrenal gland (Cao *et al.*, 1998; Hyndman and Flynn, 1998). This protein is not expressed in the lung and breast and is at a low level in the liver, but overexpressed in hepatocellular carcinoma and non-small cell lung cancer, detoxifying intracellular electrophilic carbonyls (Cao *et al.*, 1998; Fukumoto *et al.*, 2005; Zhong *et al.*, 2009). AKR1B10 is also active toward daunorubicin (Martin *et al.*, 2006; Balendiran, 2009), but its activity to other anthracycline derivatives is unknown. The action mechanism of AKR1B10 on daunorubicin (e.g. reactive chemical group) remains to be defined and its effect on cellular sensitivity of anthracyclines is unclear. In this present study, we demonstrated that AKR1B10 catalyzes the reduction of C13-ketonic group to hydroxyl form using liquid chromatograph-mass spectrometry (LC-MS) and determined its kinetic constants toward daunorubicin, idarubicin, doxorubicin, and epirubicin. We also measured AKR1B10's reductive activity toward daunorubicin and idarubicin at pharmacological concentrations and estimated the effect of AKR1B10 expression on their cellular metabolism and cell

sensitivity. The results are informative for the clinical use of anthracyclines in cancer patients.

Materials and methods

Reagents

Daunorubicin, idarubicin, epirubicin, doxorubicin, NADPH, acetonitrile, formic acid and fetal bovine serum (FBS) were purchased from Sigma, MO. Penicillin, streptomycin, trypsin, and DMEM were purchased from Fisher, FL.

Cell culture and transient transfection

Transformed human embryonic kidney cells (293T) and human non-small cell lung cancer cells (NCI-H460) were purchased from American Type Culture Collection (Manassas, VA) and grown in DMEM (for 293T) or RPMI-1640 (for NCI-H460) medium (Hyclone, UT) supplemented with 10% FBS and 1% penicillin/streptomycin solution. Enhanced green fluorescent protein (EGFP)-AKR1B10 expression vector was constructed as described previously (Zu *et al.*, 2007). Transfections were performed using ExGen-500 (Fermentas, MD) at 80% of cell confluence, following manufacturer's instructions. Expression and functionality of the EGFP-AKR1B10 fusion protein were verified by Western blot and enzyme activity. EGFP-C3 vector was introduced into 293T cells in parallel as a control.

AKR1B10 purification and activity assay

AKR1B10 recombinant protein was prepared using a pQE prokaryotic protein expression system (Qiagen, CA) as previously described (Zhong *et al.*, 2009). Enzyme activity was measured at 35°C for 20 min in 500 µl of the reaction mixture containing 100 mM Tris.Cl (pH 7.4), 50 mM KCl, 200 µM NADPH, 2 µg purified AKR1B10 protein, and appropriate substrates. Blank controls contained all components of the reaction mixture except for the protein. Enzymatic products were analyzed by HPLC or LC-MS (see below). Standards of daunorubicinol, idarubicinol, doxorubicinol, and epirubicinol were synthesized by complete reduction with excessive AKR1B1 proteins (50 µg). Kinetic constants (K_m and V_{max}) were calculated with GraphPad Prism 4 (Graph Pad Software, CA). $k_{cat} = V_{max}/[E]$. [E] denotes enzyme concentrations in molar.

High-performance liquid chromatography (HPLC)

Enzyme reaction mixture was filtered with a 5 kDa filter to remove protein and other macromolecules and diluted with 5 mM ammonium acetate/acetonitrile, if needed. Enzymatic products were achieved using a HPLC system equipped with a dual fluorescent detector (Shimadzu, Japan). A premier C18 column (4.0 × 250 mm, 5 µm particles; Shimadzu, Japan), protected by a pre-column with the same materials, was used for separation. Mobile phase consisted of a mixture of Buffer A (50 mM acetate sodium in deionized water at pH 4.0, adjusted with formic acid) and 25–35% Buffer B (acetonitrile; Sigma, MO). Flow rate was at 0.8–1.0 ml/min. Eluents were detected with 480nm for excitation and 542nm for emission.

Liquid chromatograph-mass spectrometry (LC-MS)

Enzymatic products were purified and diluted as above for HPLC analysis. For tandem LC-MS analysis, a micromass triple quadrupole mass spectrometer (Waters, MA) was used that is operated in a positive ionization mode with a unit mass resolution. Chromatographic separations were performed with a reversed phase, C18 column (2 × 50 mm, 5 µm; Waters, MA) at 0.2 ml/min of gradient acetonitrile (5% to 95% within 20 min). Resulting ions were first monitored using a selective ion recording (SIR) mode. Ion transitions, m/z 528.1 (MH^+)

to 321.1 and 530.1 to 321.1 or 323.1 were monitored in a multiple reaction monitoring (MRM, second order MS) mode. Capillary and cone voltages were set at 4.3 kV and 33 V; source and desolvation temperatures were 120°C and 325°C, respectively. Electron spray gas was provided with a high pressured liquid nitrogen tank. For MRM, argon of ultra high purity was used as the collision gas.

Anthracycline intracellular metabolism

To test the metabolism at low concentrations, 293T cells transfected with EGFP-AKR1B10 or control vector EGFP-C3 were collected after transfection for 36 hours and suspended at 1×10^6 cells/ml. Daunorubicin (50nM) or idarubicin (30nM) was added for 1 hour and then cells were subjected to extraction of metabolites as below. To observe metabolic kinetics, transfected 293T cells were seeded in 24-well plates at 5×10^4 cells/well overnight and then exposed to 1 μ M of daunorubicin or idarubicin. Medium and cells were collected at indicated time points during a 24-hour's period. For extraction of metabolites, cells were frozen and thawed for 4 cycles and then sonicated on ice at 5 sec for 4 bursts at 200W with a 10 sec interval each. Cell lysates were vortexed with acetonitrile vigorously, and the aqueous phase was collected at 14,000 rpm, 4°C for 10 min and lyophilized. After being dissolved in 100 μ l dH₂O, 85 μ l was uploaded for HPLC analysis as described above.

MTT assay

Idarubicin and daunorubicin cytotoxicity was assayed by MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole]. Briefly, cells (5,000 cells/well) were seeded in 96-well plates overnight and then fed with 100 μ l of fresh medium containing drugs at indicated concentrations for 72 hours. After being exposed to MTT for 4 hours, 100 μ l of DMSO was added to each well and the absorbance at 570nm was read. Cell viability was presented as percentage of control cells. AKR1B10 inhibitor epalrestat (Wang *et al.*, 2009) was used at 100 μ M in another study to test its effect on daunorubicin and idarubicin cytotoxicity.

Statistical analysis

Student *t* tests or Chi-square tests of independence, as appropriate, were used for statistically significant tests of data with $p < 0.05$.

Results

AKR1B10 reduces C13-ketonic group in daunorubicin and idarubicin

Recombinant AKR1B10 protein was purified homogeneously and its enzyme activity was verified with DL-glyceraldehyde as a substrate (Figure 1S). This protein was then used to catalyze reduction of daunorubicin and idarubicin. As shown in Figure 1A, a peak with retention time of 7.93 min (marked with X) was detected in daunorubicin reaction mixture. This peak was well separated from daunorubicin's peak at 8.51 min and approximately 30 times higher in daunorubicin-AKR1B10 reactant mixture than in AKR1B10-free control. This peak had an ion transition of m/z 530.1, indicating addition of two hydrogen protons to parental daunorubicin (m/z 528.1). Therefore, this peak may represent the reduced products of daunorubicin.

An MRM study that can pinpoint chemical structures confirmed this finding. Figure 1B shows that after collision, this m/z 530.1 product gave an ion transition of m/z 530.1 to 321.1, rather than 323.1, suggesting that the addition of hydrogen protons occurred at C13-ketonic group on the side chain of daunorubicin, producing daunorubicinol. Similar results were obtained in idarubicin (data not shown).

AKR1B10 prefers for daunorubicin and idarubicin with a C14-methyl group

Doxorubicin, epirubicin, and idarubicin are three major derivatives of daunorubicin, used for cancer treatment. As shown in Figure 2S, idarubicin is derived from daunorubicin by removal of the methoxy group at C4, whereas doxorubicin is different from daunorubicin at the C14 group, having a C14-hydroxyl side chain. Epirubicin is a derivative of doxorubicin by an axial-to-equatorial epimerization of the hydroxyl group at C-4 of ribose ring (Menna *et al.*, 2007). Steady-state kinetic parameters for daunorubicin and these derivatives were determined by measuring their reduction products at different substrate concentrations. Figure 2 displays substrate-velocity curves and Lineweaver-Burk plots (in frame) of these agents. The kinetic constants are summarized in Table 1. The data showed that AKR1B10 had appreciable catalytic activity to daunorubicin and idarubicin with a C14-methyl group on their side chain, but not to doxorubicin and epirubicin, suggesting that the C14-hydroxyl group significantly affects their substrate specificity. In addition, compared to daunorubicin, the removal of C4-methoxy group in idarubicin lowered down its K_m values for approximate 20 folds and increased the product turn-over rate (k_{cat}/K_m) for about 10 times. The axial-to-equatorial epimerization of hydroxyl group at C-4 appeared to have negligible effect on the substrate specificity. It is noteworthy that daunorubicin seemed to fit better the hill equation, consistent with the report by Martin and colleagues (Martin *et al.*, 2006). Idarubicin, as well as doxorubicin and epirubicin (although lower substrate specificity), demonstrated a Michaelis-Menten hyperbola.

To understand the pharmacological significance of this AKR1B10-catalyzed reduction of daunorubicin and idarubicin, we examined their substrate specificity at low concentrations. As shown in Figure 3, AKR1B10 converted approximately 14% of daunorubicin at 50nM and 26% of idarubicin at 30nM to their corresponding alcoholic forms, daunorubicinol and idarubicinol, at experimental conditions, indicating AKR1B10's activity toward these two agents at pharmacological levels that are high up to 200 – 300nM in patient plasma (Reid *et al.*, 1990; Galettis *et al.*, 1994).

Ectopically expressed AKR1B10 participates in intracellular metabolism of daunorubicin and idarubicin

In vitro enzymatic studies indicated AKR1B10's role in idarubicin and daunorubicin metabolism. To confirm its role inside cells, we ectopically expressed AKR1B10 in 293T cells. To avoid compensative alterations of cells in response to AKR1B10 delivery, transient transfection was used in this study. An EGFP was fused to the N-terminus of AKR1B10 to indicate the transfection efficiency. As shown in Figure 3S, this 65.0 kDa EGFP-AKR1B10 fusion protein is enzymatically active to DL-glyceraldehyde. Using the AKR1B10 expression and vector control cells, we first estimated its reductive activity toward daunorubicin (50nM) and idarubicin (30nM), the substrate concentration used *in vitro*. As demonstrated in Figure 4, ectopically expressed AKR1B10 in 293T cells catalyzed the production of idarubicinol and daunorubicinol, compared to vector control. This data suggests the role of AKR1B10 in cellular metabolism of daunorubicin and idarubicin at pharmacological conditions.

We further examined dynamic production of daunorubicinol and idarubicinol in AKR1B10 expression cells. In this study, daunorubicin or idarubicin was used at a low concentration of 1 μ M for a 24-hour's period. At indicated time points, medium and cells were collected to measure daunorubicinol or idarubicinol produced. As shown in Figure 5, conversion of daunorubicin or idarubicin to alcoholic forms was significantly higher in 293T cells with AKR1B10 expression than in vector control cells. Within 24 hours, nearly 20 $\pm$ 2.7% of daunorubicin was reduced to daunorubicinol compared to approximate 7 $\pm$ 0.9% in vector control ($p < 0.05$). The conversion of idarubicin to idarubicinol was at 23 $\pm$ 2.3% in

AKR1B10 expression cells and $5 \pm 1.5\%$ in the vector control ($p < 0.05$). This data suggests that AKR1B10 indeed participates in the intracellular metabolism of idarubicin and daunorubicin.

AKR1B10 induces cell resistance to daunorubicin and idarubicin

Reduction of the C13-ketonic group to alcoholic forms lowers down the cytotoxicity of anthracyclines. Therefore, we further evaluated the effect of AKR1B10 expression on the cytotoxicity of daunorubicin and idarubicin. To better address this question, two approaches were carried out, i.e., ectopic expression of AKR1B10 and inhibition of endogenous AKR1B10 activity by inhibitor epalrestat. The 293T cells that do not express AKR1B10 were used for the ectopic expression of AKR1B10, and empty vector was used as a control. Human lung cancer cells (NCI-H460) that express AKR1B10 but not aldose reductase (Wang *et al.*, 2009) were used for the inhibitory study. After the cells were exposed to daunorubicin and idarubicin at indicated concentrations for 72 hours, viable cells were measured using MTT assays. As shown in Figure 6, AKR1B10 expression noticeably decreased cell sensitivity to idarubicin and daunorubicin whereas inhibition of AKR1B10 activity by epalrestat significantly sensitized NCI-H460 cells to these two drugs. These data indicate that AKR1B10 may induce cell resistance to daunorubicin and idarubicin.

Discussion

Resistance to anticancer drugs is a major drawback of cancer chemotherapy, leading to treatment failure (Hembruff *et al.*, 2008). AKR1B10 is overexpressed in several human tumors, such as the liver and lung cancer (Cao *et al.*, 1998; Fukumoto *et al.*, 2005), and has a broad substrate specificity to xenobiotics, including antitumor agents containing carbonyl groups (Jin and Penning, 2007; Barski *et al.*, 2008). Anthracyclines are the most effective anticancer agents used in the treatment of lymphoma, leukemia, and lung, liver, and breast cancers. However, resistance of cancer cells to anthracyclines occurs frequently. This study investigated the enzymatic activity of AKR1B10 toward four major anthracyclines (daunorubicin, idarubicin, doxorubicin and epirubicin) and evaluated its role in intracellular metabolism and cytotoxicity of daunorubicin and idarubicin. The study results recognized AKR1B10 as a novel factor for cell resistance to daunorubicin and idarubicin by reducing them to alcoholic forms.

Anthracyclines contain a C13-ketonic group on the side chain. All anthracyclines share this functional C13 group, but vary on C14 group of this side chain, which is a methyl group in daunorubicin and idarubicin, but is a hydroxyl group in doxorubicin and epirubicin (Figure 2S). This alteration may perceptibly change the polarity of the side chain, leading to the differential substrate specificity toward AKR1B10. Clearly, this study showed that daunorubicin and idarubicin were much more appreciable substrates of AKR1B10, *in vitro* and inside cells, than doxorubicin and epirubicin. It has been reported that native AKR1B10 has enzyme activity to daunorubicin (Martin *et al.*, 2006). This study evidently confirmed C13-ketonic group on side chain of daunorubicin and idarubicin as the active site of AKR1B10 using LC-MS/MS that can pinpoint the chemical structure. Of note, in our study, the recombinant AKR1B10 protein showed K_m at 9.32 ± 2.25 mM and k_{cat}/K_m at 3.24 to daunorubicin, which are higher than the K_m of 1.1 ± 0.18 mM and k_{cat}/K_m of 1.3 for the native AKR1B10 reported by Martin, *et al.* (Martin *et al.*, 2006). This data supports their suggestion that native AKR1B10 has higher catalytic efficiency. Interestingly, our study revealed that idarubicin is much better a substrate of AKR1B10, with K_m at 0.461 ± 0.09 mM and k_{cat}/K_m at 35.94. This indicates that the methoxy group at C4 may affect their substrate specificity. Idarubicin is pervasively used for the treatment of lung cancer, lymphoma, and leukemia (Ohtake *et al.*); therefore, this finding would have important clinical implication. The C14-methyl group on the side chain of doxorubicin and epirubicin significantly affects

their substrate specificity, but same to idarubicin, doxorubicin and epirubicin both showed Michaelis-Menten kinetic properties. Daunorubicin appeared to be better fitted by the Hill equation, consistent with the report of Martin, et al (Martin *et al.*, 2006). It is noteworthy that in all kinetic assays, substrate concentrations were started from 0.1mM until the plateau of enzyme activity or presence of slight substrate inhibition. Therefore, differences of substrate concentrations in Figure 2 indicate the differentials in plateau.

Drug resistance and cardiotoxicity induced by reduction products of anthracyclines such as daunorubicinol limit their success in cancer patients (Shan *et al.*, 1996). Previous studies have shown that daunorubicinol with a C13-hydroxyl group has significantly less potency in inhibiting tumor cell growth than its parental form daunorubicin, but stronger cardiomyopathic toxicity (Beran *et al.*, 1979; Kuffel *et al.*, 1992; Ohara *et al.*, 1995; Soldan *et al.*, 1999). To evaluate the pharmacological significance, AKR1B10's reduction activity towards daunorubicin and idarubicin was tested at low concentrations, 50nM for daunorubicin and 30nM for idarubicin. In clinical patients, daunorubicin administrated at a single dose of 50 mg/m² (i.v.) reaches a peak concentration of more than 300nM in plasma (Galettis *et al.*, 1994). Peak plasma concentration of idarubicin at a single dose of 10 mg/m² (i.v.) is up to 180nM (Reid *et al.*, 1990). Therefore, our data of AKR1B10 activity toward daunorubicin and idarubicin suggests its pharmacological role in the metabolism and cytotoxicity of these drugs. This was further evidenced by a cell sensitivity study. Inside living cells 293T, daunorubicin and idarubicin were efficiently reduced to corresponding alcoholic forms by ectopically expressed AKR1B10, leading to cell resistance to these two agents.

A great number of aldose reductase inhibitors (ARIs), such as epalrestat, have been developed and used in diabetic clinics (Krentz *et al.*, 1992; Hotta *et al.*, 1996; Asano *et al.*, 2002; Giannoukakis, 2008; Bril *et al.*, 2009). Interestingly, some ARIs have strong inhibitory activity to AKR1B10 and induce apoptotic cell death (Gallego *et al.*, 2007; Verma *et al.*, 2008; Wang *et al.*, 2009; Shen *et al.*, 2010). This study showed that daunorubicin and idarubicin and epalrestat displayed synergistic role in suppressing cell growth and viability, indicating a potential new combination of drugs in cancer treatment.

In summary, this work presented essential evidence that AKR1B10 catalyzes reduction of daunorubicin and idarubicin to alcoholic metabolites *in vitro* and inside cells. This study defined the active group in anthracyclines that AKR1B10 works on and estimated the effect of AKR1B10 expression on intracellular metabolism and cell sensitivity of daunorubicin and idarubicin. The data suggests that AKR1B10 overexpressed in breast, liver and lung cancer may lead to tumor resistance to daunorubicin and idarubicin. This study result is informative for the use of these agents in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Cancer Institute [CA122327].

Abbreviations used

AKR1B10	aldo-keto reductase family 1 B10
EGFP	enhanced green fluorescent protein

ESI	electrospray ionization; 4-hydroxynonenal
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography-mass spectroscopy
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

References

- Asano T, Saito Y, Kawakami M, Yamada N. Fidarestat (SNK-860), a potent aldose reductase inhibitor, normalizes the elevated sorbitol accumulation in erythrocytes of diabetic patients. *J Diabetes Complications*. 2002; 16:133–138. [PubMed: 12039395]
- Asbell MA, Schwartzbach E, Bullock FJ, Yesair DW. Daunomycin and adriamycin metabolism via reductive glycosidic cleavage. *The Journal of pharmacology and experimental therapeutics*. 1972; 182:63–69. [PubMed: 5041655]
- Balendiran GK. Fibrates in the chemical action of daunorubicin. *Current cancer drug targets*. 2009; 9:366–369. [PubMed: 19442055]
- Barski OA, Tipparaju SM, Bhatnagar A. The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metab Rev*. 2008; 40:553–624. [PubMed: 18949601]
- Beran M, Andersson B, Eksborg S, Ehrsson H. Comparative studies on the in vitro killing of human normal and leukemic clonogenic cells (CFUc) by daunorubicin, daunorubicinol, and daunorubicin-DNA complex. *Cancer Chemother Pharmacol*. 1979; 2:19–24. [PubMed: 498414]
- Bril V, Hirose T, Tomioka S, Buchanan R. Ranirestat for the Management of Diabetic Sensorimotor Polyneuropathy. *Diabetes Care*. 2009
- Cao D, Fan ST, Chung SS. Identification and characterization of a novel human aldose reductase-like gene. *J Biol Chem*. 1998; 273:11429–11435. [PubMed: 9565553]
- Chung S, LaMendola J. Cloning and sequence determination of human placental aldose reductase gene. *J Biol Chem*. 1989; 264:14775–14777. [PubMed: 2504709]
- Den Boer ML, Pieters R, Veerman AJ. Mechanisms of cellular anthracycline resistance in childhood acute leukemia. *Leukemia*. 1998; 12:1657–1670. [PubMed: 9823938]
- Dimarco A, Gaetani M, Orezzi P, Scarpinato BM, Silvestrini R, Soldati M, Dasdia T, Valentini L. ‘Daunomycin’, a New Antibiotic of the Rhodomycin Group. *Nature*. 1964; 201:706–707. [PubMed: 14142092]
- Felsted RL, Bachur NR. Human liver daunorubicin reductases. Progress in clinical and biological research. 1982; 114:291–305. [PubMed: 6761708]
- Fukumoto S, Yamauchi N, Moriguchi H, Hippo Y, Watanabe A, Shibahara J, Taniguchi H, Ishikawa S, Ito H, Yamamoto S, Iwanari H, Hironaka M, Ishikawa Y, Niki T, Sohara Y, Kodama T, Nishimura M, Fukayama M, Dosaka-Akita H, Aburatani H. Overexpression of the aldo-keto reductase family protein AKR1B10 is highly correlated with smokers’ non-small cell lung carcinomas. *Clin Cancer Res*. 2005; 11:1776–1785. [PubMed: 15755999]
- Galettis P, Boutagy J, Ma DD. Daunorubicin pharmacokinetics and the correlation with P-glycoprotein and response in patients with acute leukaemia. *Br J Cancer*. 1994; 70:324–329. [PubMed: 7914424]
- Gallego O, Ruiz FX, Ardevol A, Dominguez M, Alvarez R, de Lera AR, Rovira C, Farres J, Fita I, Pares X. Structural basis for the high all-trans-retinaldehyde reductase activity of the tumor marker AKR1B10. *Proc Natl Acad Sci U S A*. 2007; 104:20764–20769. [PubMed: 18087047]
- Gavelova M, Hladikova J, Vildova L, Novotna R, Vondracek J, Krcmar P, Machala M, Skalova L. Reduction of doxorubicin and oracin and induction of carbonyl reductase in human breast carcinoma MCF-7 cells. *Chemico-biological interactions*. 2008; 176:9–18. [PubMed: 18755171]
- Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol*. 1999; 57:727–741. [PubMed: 10075079]

- Gianni L. Anthracycline resistance: the problem and its current definition. *Semin Oncol.* 1997; 24:S10–11–S10–17. [PubMed: 9275001]
- Giannoukakis N. Ranirestat as a therapeutic aldose reductase inhibitor for diabetic complications. *Expert Opin Investig Drugs.* 2008; 17:575–581.
- Gottesman MM. Mechanisms of cancer drug resistance. *Annual review of medicine.* 2002; 53:615–627.
- Hembruff SL, Laberge ML, Villeneuve DJ, Guo B, Veitch Z, Cecchetto M, Parissenti AM. Role of drug transporters and drug accumulation in the temporal acquisition of drug resistance. *BMC cancer.* 2008; 8:318. [PubMed: 18980695]
- Hotta N, Sakamoto N, Shigeta Y, Kikkawa R, Goto Y. Clinical investigation of epalrestat, an aldose reductase inhibitor, on diabetic neuropathy in Japan: multicenter study. *Diabetic Neuropathy Study Group in Japan. J Diabetes Complications.* 1996; 10:168–172. [PubMed: 8807467]
- Hussein MA. Preclinical rationale, mechanisms of action, and clinical activity of anthracyclines in myeloma. *Clin Lymphoma Myeloma.* 2007; 7(Suppl 4):S145–149. [PubMed: 17562252]
- Hyndman D, Bauman DR, Heredia VV, Penning TM. The aldo-keto reductase superfamily homepage. *Chem Biol Interact.* 2003; 143–144:621–631.
- Hyndman DJ, Flynn TG. Sequence and expression levels in human tissues of a new member of the aldo-keto reductase family. *Biochim Biophys Acta.* 1998; 1399:198–202. [PubMed: 9765596]
- Jez JM, Flynn TG, Penning TM. A nomenclature system for the aldo-keto reductase superfamily. *Adv Exp Med Biol.* 1997; 414:579–600. [PubMed: 9059665]
- Jin Y, Penning TM. Aldo-keto reductases and bioactivation/detoxication. *Annu Rev Pharmacol Toxicol.* 2007; 47:263–292. [PubMed: 16970545]
- Krentz AJ, Honigsberger L, Ellis SH, Hardman M, Nattrass M. A 12-month randomized controlled study of the aldose reductase inhibitor ponalrestat in patients with chronic symptomatic diabetic neuropathy. *Diabet Med.* 1992; 9:463–468. [PubMed: 1611835]
- Kuffel MJ, Reid JM, Ames MM. Anthracyclines and their C-13 alcohol metabolites: growth inhibition and DNA damage following incubation with human tumor cells in culture. *Cancer Chemother Pharmacol.* 1992; 30:51–57. [PubMed: 1586980]
- Lee KW, Ko BC, Jiang Z, Cao D, Chung SS. Overexpression of aldose reductase in liver cancers may contribute to drug resistance. *Anticancer Drugs.* 2001; 12:129–132. [PubMed: 11261885]
- Liu Z, Zhong L, Krishack PA, Robbins S, Cao JX, Zhao Y, Chung S, Cao D. Structure and promoter characterization of aldo-keto reductase family 1 B10 gene. *Gene.* 2009; 437:39–44. [PubMed: 19236911]
- Martin HJ, Breyer-Pfaff U, Wsol V, Venz S, Block S, Maser E. Purification and characterization of akr1b10 from human liver: role in carbonyl reduction of xenobiotics. *Drug Metab Dispos.* 2006; 34:464–470. [PubMed: 16381663]
- Menna P, Minotti G, Salvatorelli E. In vitro modeling of the structure-activity determinants of anthracycline cardiotoxicity. *Cell biology and toxicology.* 2007; 23:49–62. [PubMed: 17031515]
- Moretti E, Oakman C, Di Leo A. Predicting anthracycline benefit: have we made any progress? *Curr Opin Oncol.* 2009; 21:507–515. [PubMed: 19713842]
- Muggia FM, Green MD. New anthracycline antitumor antibiotics. *Crit Rev Oncol Hematol.* 1991; 11:43–64. [PubMed: 1831987]
- Murphy, GP.; Lawrence, W., Jr; Lenhard, RE. *American Society Textbook of Clinical Oncology.* 2. 1995.
- Niitsu N, Kasukabe T, Yokoyama A, Okabe-Kado J, Yamamoto-Yamaguchi Y, Umeda M, Honma Y. Anticancer derivative of butyric acid (Pivalyloxymethyl butyrate) specifically potentiates the cytotoxicity of doxorubicin and daunorubicin through the suppression of microsomal glycosidic activity. *Molecular pharmacology.* 2000; 58:27–36. [PubMed: 10860924]
- Ohara H, Miyabe Y, Deyashiki Y, Matsuura K, Hara A. Reduction of drug ketones by dihydrodiol dehydrogenases, carbonyl reductase and aldehyde reductase of human liver. *Biochemical pharmacology.* 1995; 50:221–227. [PubMed: 7632166]
- Ohtake S, Miyawaki S, Fujita H, Kiyoi H, Shinagawa K, Usui N, Okumura H, Miyamura K, Nakaseko C, Miyazaki Y, Fujieda A, Nagai T, Yamane T, Taniwaki M, Takahashi M, Yagasaki F, Kimura Y, Asou N, Sakamaki H, Handa H, Honda S, Ohnishi K, Naoe T, Ohno R. Randomized study of

- induction therapy comparing standard-dose idarubicin with high-dose daunorubicin in adult patients with previously untreated acute myeloid leukemia: JALSG AML201 Study. *Blood*. Petrash JM. All in the family: aldose reductase and closely related aldo-keto reductases. *Cell Mol Life Sci*. 2004; 61:737–749. [PubMed: 15094999]
- Plebuch M, Soldan M, Hungerer C, Koch L, Maser E. Increased resistance of tumor cells to daunorubicin after transfection of cDNAs coding for anthracycline inactivating enzymes. *Cancer letters*. 2007; 255:49–56. [PubMed: 17482758]
- Reid JM, Pendergrass TW, Krailo MD, Hammond GD, Ames MM. Plasma pharmacokinetics and cerebrospinal fluid concentrations of idarubicin and idarubicinol in pediatric leukemia patients: a Childrens Cancer Study Group report. *Cancer Res*. 1990; 50:6525–6528. [PubMed: 2208112]
- Schroterova L, Kaiserova H, Baliharova V, Velik J, Gersl V, Kvasnickova E. The effect of new lipophilic chelators on the activities of cytosolic reductases and P450 cytochromes involved in the metabolism of anthracycline antibiotics: studies in vitro. *Physiological research/Academia Scientiarum Bohemoslovaca*. 2004; 53:683–691. [PubMed: 15588138]
- Shan K, Lincoff AM, Young JB. Anthracycline-induced cardiotoxicity. *Ann Intern Med*. 1996; 125:47–58. [PubMed: 8644988]
- Shen Y, Zhong L, Markwell S, Cao D. Thiol-disulfide exchanges modulate aldo-keto reductase family 1 member B10 activity and sensitivity to inhibitors. *Biochimie*. 2010; 92:530–537. [PubMed: 20144905]
- Soldan M, Ax W, Plebuch M, Koch L, Maser E. Cytostatic drug resistance. Role of phase-I daunorubicin metabolism in cancer cells. *Adv Exp Med Biol*. 1999; 463:529–538. [PubMed: 10352729]
- Verma M, Martin HJ, Haq W, O'Connor TR, Maser E, Balendiran GK. Inhibiting wild-type and C299S mutant AKR1B10; a homologue of aldose reductase upregulated in cancers. *Eur J Pharmacol*. 2008; 584:213–221. [PubMed: 18325492]
- Wang C, Yan R, Luo D, Watabe K, Liao DF, Cao D. Aldo-keto reductase family 1 member B10 promotes cell survival by regulating lipid synthesis and eliminating carbonyls. *J Biol Chem*. 2009; 284:26742–26748. [PubMed: 19643728]
- Wermuth B, Bohren KM, Heinemann G, von Wartburg JP, Gabbay KH. Human carbonyl reductase. Nucleotide sequence analysis of a cDNA and amino acid sequence of the encoded protein. *The Journal of biological chemistry*. 1988; 263:16185–16188. [PubMed: 3141401]
- Wiernik PH, Dutcher JP. Clinical importance of anthracyclines in the treatment of acute myeloid leukemia. *Leukemia*. 1992; 6(Suppl 1):67–69. [PubMed: 1548939]
- Zhong L, Liu Z, Yan R, Johnson S, Zhao Y, Fang X, Cao D. Aldo-keto reductase family 1 B10 protein detoxifies dietary and lipid-derived alpha, beta-unsaturated carbonyls at physiological levels. *Biochem Biophys Res Commun*. 2009; 387:245–250. [PubMed: 19563777]
- Zu X, Yan R, Robbins S, Krishack PA, Liao DF, Cao D. Reduced 293T cell susceptibility to acrolein due to aldose reductase-like-1 protein expression. *Toxicol Sci*. 2007; 97:562–568. [PubMed: 17329238]

Highlights

1. This study defines the enzymatic activity of AKR1B10 protein towards all four most frequently used antitumor anthracyclines: daunorubicin, idarubicin, doxorubicin, and epirubicin.
2. This study pinpoints the chemical group—C13 ketone that AKR1B10 acts on, and identifies that the C14 group (methyl or hydroxyl) in these agents affects their substrate specificity towards AKR1B10.
3. This study defines the role of AKR1B10 in cellular metabolism and pharmacokinetics of these anthracyclines
4. This study confirms the role of AKR1B10 in the cell resistance to anthracyclines by both ectopic expression of AKR1B10 and its inhibitor epalrestat in an *in vitro* cell culture model. The study results may be relevant for the clinical application of anthracyclines in cancer patients.

A) Selective ion recording mode

B) Multiple reaction monitoring mode

Figure 1. Liquid chromatography-mass spectrometry (LC-MS) of daunorubicinol

Enzyme reaction with 10 μ M of daunorubicin as a substrate and LC-MS were conducted as described in the Materials and Methods. (A) Selective ion recording (SIR) mode. A peak (daunorubicinol, marked with x) with m/z 530.1 was detected by 30 times higher in AKR1B10 reaction mixture (upper) than in AKR1B10-free control (lower), indicating addition of two hydrogen protons. This m/z 530.1 peak has a retention time of 7.93 min and was well separated from daunorubicin (m/z 528.1) at 8.51 min. (B) Multiple reaction monitoring (MRM) mode. This mode confirmed the presence of reductive products with m/z 530.1 (upper) and showed an ion transition of m/z 530.1 to m/z 321.1, rather than m/z 323.1, indicating that this reduction occurred on the C13-ketonic group of side chain and produced daunorubicinol. DA, daunorubicin.

A) Daunorubicin

B) Idarubicin

C) Doxorubicin

D) Epirubicin

Figure 2. Substrate-velocity curves of anthracyclines

Enzymatic reactions with various anthracyclines at different concentrations as indicated were conducted and reaction mixtures were analyzed by high-performance liquid chromatography as described in the Materials and Methods. Substrate-velocity curves were produced with GraphPad Prism 4 (Graph Pad Software, CA). (A) Daunorubicin, (B) Idarubicin, (C) Doxorubicin, and (D) Epirubicin. In frame, Lineweaver-Burk plots.

A) Daunorubicin **B) Idarubicin**

Figure 3. AKR1B10 activity toward daunorubicin (A) and idarubicin (B) at low concentrations Enzyme reactions with daunorubicin (50nM) and idarubicin (30nM) were conducted and reaction mixtures were analyzed by high-performance liquid chromatography as described in the Materials and Methods. Acetonitrile was used at 35% in separation. Graphics show the production of daunorubicinol and idarubicinol. The peaks at retention time of approximately 3 min are sample loading noise. DNR, daunorubicin; DNRol, daunorubicinol; IDA, idarubicin; and IDAol, idarubicinol.

A) Daunorubicin

(i) Vector

(ii) EGFP-AKR1B10

B) Idarubicin

(i) Vector

(ii) EGFP-AKR1B10

Figure 4. Intracellular reduction by AKR1B10 of daunorubicin (A) and idarubicin (B) at low concentration

EGFP-AKR1B10 protein delivery, daunorubicin (50nM) and idarubicin (30nM) treatment and high-performance liquid chromatography separation were conducted as described in the Materials and Methods. Product peaks are labeled as indicated. Acetonitrile was used at 25% for better separation. The peak at approximate 9.0 min in both daunorubicin and idarubicin extracts represents an unknown compound from cells. DNR, daunorubicin; DNRol, daunorubicinol; IDA, idarubicin; and IDAol, idarubicinol.

Figure 5. Time courses of intracellular daunorubicinol and idarubicinol production

EGFP-AKR1B10 and vector control 293T cells were spread into 24-well plates and exposed to daunorubicin or idarubicin at 1 μ M. At indicated time points, cells and medium were collected for daunorubicinol and idarubicinol analysis as described in the Materials and Methods. Data represents mean $\pm$ SD from three independent experiments. * and # $p < 0.05$, compared to the relevant control. DNRol, daunorubicinol and IDAol, idarubicinol.

Figure 6. Cell sensitivity to daunorubicin and idarubicin

Cells were spread into 96-well plates and exposed to daunorubicin or idarubicin indicated for 72 hours. Viable cells were detected by a MTT cell proliferation kit as described in the Materials and Methods. (A) 293T cells transfected with EGFP-AKR1B10 or vector control. (B) NCI-H460 cells. Epalrestat was used at 100 μ M. Data represents mean $\pm$ SD from three independent experiments. Epal, epalrestat. * $p < 0.05$ and ** $p < 0.01$, compared to vector control.

Table 1

Kinetic constants of AKR1B10 toward anthracyclines.

Substrates	AKR1B10			
	V_{max} (nmol/mg/min)	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (min ⁻¹ mM ⁻¹)
Daunorubicin	837.42 ± 81.39	9.317 ± 2.25	30.15 ± 2.93	3.24
Idarubicin	460.23 ± 28.12	0.461 ± 0.09	16.57 ± 1.07	35.94
Doxorubicin	20.13 ± 2.21	0.274 ± 0.08	0.73 ± 0.06	2.66
Epirubicin	9.16 ± 0.58	0.156 ± 0.03	0.33 ± 0.02	2.12