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ORIGINAL ARTICLE

Jan S. Moreb · Dagmara Muhoczy · Blanca Ostmark
James R. Zucali

RNAi-mediated knockdown of aldehyde dehydrogenase class-1A1 and class-3A1 is specific and reveals that each contributes equally to the resistance against 4-hydroperoxycyclophosphamide

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Abstract *Purpose*: Aldehyde dehydrogenases class-1A1 (ALDH1A1) and class-3A1 (ALDH3A1) have been associated with resistance to cyclophosphamide (CP) and its derivatives. We have previously reported the downregulation of these enzymes by all-trans retinoic acid (ATRA). *Methods*: In this study, we used siRNA duplexes as well as retrovirally expressed siRNA to knockdown one or both enzymes together in A549 lung cancer cell line in order to investigate the role of each one in mediating the resistance and the effect of the addition of ATRA. *Results*: The results show that significant and specific knockdown of each enzyme can be achieved and that each one contributes similarly to cell resistance to 4-hydroperoxycyclophosphamide (4-HC), an active derivative of CP. Added effects were seen when both enzymes were inhibited. The addition of ATRA also exhibited additional inhibitory effects on ALDH activity and increased 4-HC toxicity when added to single siRNA aimed at one of the enzymes. On the other hand, ATRA had minimal and insignificant additional inhibitory effects on ALDH enzyme activity when added to a combination of siRNAs against both enzymes, but still increased 4-HC toxicity beyond that seen with RNAi-mediated inhibition of both enzymes together. *Conclusions*: We conclude that both enzymes, ALDH1A1 and ALDH3A1 will need to be blocked in order to achieve the highest sensitivity to 4-HC. Furthermore, ATRA increases 4-HC toxicity even when added to a combination of siRNAs against both enzymes, thus suggesting additional mechanisms by which ATRA can increase drug toxicity.

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J. S. Moreb (✉) · D. Muhoczy · B. Ostmark · J. R. Zucali
Department of Medicine, Division of Hematology/Oncology, University of Florida, College of Medicine, 1600 SW Archer Road, Room R4-220, PO Box 100277, Gainesville, FL 32610, USA
E-mail: morebjs@medicine.ufl.edu
Tel.: +1-352-3923875
Fax: +1-352-3922323

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Introduction

Aldehyde dehydrogenases (ALDH) are a group of enzymes catalyzing the conversion of a broad range of aldehydes to the corresponding acid via a NAD^+-dependent irreversible reaction. Seventeen enzymes are currently viewed as belonging to the human ALDH superfamily. The molecular and biological significance of these enzymes were elegantly reviewed previously [1–3]. Two of these enzymes, cytosolic aldehyde dehydrogenase class-1A1 (ALDH1A1) and class-3A1 (ALDH3A1) have been found to be responsible for drug resistance in various tumor types against oxazaphosphorines which include cyclophosphamide [4–8]. We and others [9–13] have successfully shown that over expression of ALDH1A1 or ALDH3A1 in cell lines and in normal hematopoietic progenitor cells result in a significant increase in resistance to the active metabolites of cyclophosphamide in vitro. Furthermore, we have shown that downregulation of ALDH1A1 [14] and ALDH3A1 (unpublished data) using antisense RNA results in increased sensitivity of tumor cells to 4-hydroperoxycyclophosphamide (4-HC), an active derivative of cyclophosphamide. Our previous study showed that retinoids, in physiologic and pharmacologic doses, suppressed the levels of ALDH1A1 and ALDH3A1 proteins and enzyme activity which resulted in increased 4-HC toxicity [15]. In our opinion, the downregulation of each of these two isozymes represent not only an important strategy to reverse drug resistance but also to study the contribution of each enzyme to drug resistance determine the regulatory mechanisms involved in their cellular expression in tumor versus normal tissues.

RNA interference (RNAi) is a phenomenon whereby double-stranded RNA induces sequence-dependent deg-

radation of a cognate mRNA and shuts off cellular protein synthesis [16, 17]. This includes short interfering RNA (siRNA) which seems to be a potent and specific method for gene silencing [18–20]. Therefore, 21-nucleotide siRNA duplexes can be chemically synthesized and provide a new tool for studying gene function in mammalian cells and may eventually be used as gene-specific therapeutics [21]. In this study, we chose this approach to down regulate the ALDH1A1 and ALDH3A1 expression in lung cancer cells. We sought to test the hypothesis that the siRNA would specifically inhibit ALDH1A1 and ALDH3A1 and therefore define the role of each isozyme in the resistance to cyclophosphamide and its derivatives. Furthermore, the ability to "knockdown" both enzymes would allow us to determine whether retinoic acid increases 4-HC toxicity in vitro mainly through its inhibitory effect on their protein levels and/or through other mechanisms.

Materials and methods

Materials

All-trans RA (ATRA), NAD, and propionaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). ATRA was dissolved in ethanol. 4-HC was supplied generously by Dr. Susan Ludeman, Duke University, Durham, NC. 4-HC was freshly dissolved in culture medium for 1 μg/μl solution and filter-sterilized.

siRNA sequences

To design the siRNA for the ALDH1A1 or ALDH3A1, their sequences, as published in the GeneBank database (Accession numbers NM_000689 and NM_000691, respectively) were analyzed using the computer program through the Dharmacon's siDesign Center (http://www.dharmacon.com). Based on this approach, four oligonucleotide duplexes, two for each ALDH isozyme, were chosen. The target sequences were: 5′-TTGCTATG GCGTGGTAAGT-3′ (1A1a) and 5′-GACTTACCTGT CCTACTCA-3′ (1A1b) for the ALDH1A1, and 5′-TGA ATGGAACGCCTACTAT-3′ (3A1A) and 5′-GGTTCG ACCATATCCTGTA-3′ (3A1B) for the ALDH3A1.

Also, based on the published siGenome list (Dharmacon), the SmartPool for ALDH3A1, consisting of four pooled sequences with guaranteed silencing effect on the ALDH3A1 mRNA after 48 h, was tested in A549 cells. The separate sequences were tested as well. The target sequences were as follows: 5′-TGAGGCCTCTGATGA ATGA-3′ (D1), 5′-GAGGAGATCGAGTACATGA-3′ (D2), 5′-TAGAGGAGATCGAGTACAT-3′ (D3), 5′-TGAATGGAACGCCTACTAT-3′ (D4). Two duplexes for ALDH1A1 also available from Dharmacon were tested in A549 cells. The target sequences were: 5′-GGA CAATGCTGTTGAATTT-3′ (AD1) and 5′-CAAAGA AGCTGCCGGGTTT-3′ (AD2). Non-specific siRNA pool (Dharmacon catalogue number D-001206–13-05) was used in selected experiments for control.

In order to verify transfection efficiency with the duplexes, siRNA cassette using the 1A1a sequence in a hairpin form under the human U6 promoter and labeled with 5′-Cy3 was constructed (GeneScript). The hairpin sequence was: 5′-GGATCCCGTTGCTATGGCGTGG TAAGTTTGATATCCGACTTACCACGCCATAGCA ATTTTTTCCAAAAGCTT-3′. The A549 cells were transfected with this cassette using Oligofectamine (Olf), and epifluorescence was observed using fluorescence microscope and the number of fluorescent cells was estimated.

Cell culture and transfection

The lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in RPMI-1640 medium with 10% FBS, in 5% CO_2, 37°C cell culture incubator. The day before transfection cells were trypsynized and plated (10^5 cells/ml/well) into six-well plates for next day confluence of 40%. Cells were transfected with siRNA using Oligofectamine (Invitrogen) in Opti-MEM, according to the manufacturer protocol. Transfected and wild type cells with and without Olf were then used for the experiments described below. In some experiments as indicated, cells transfected with a pool of non-specific siRNA duplexes were used as an additional control.

Construction of siRNA expression retroviral vector

In order to explore the possibility of achieving long term and better silencing of ALDH expression, we chose to express only one of the siRNA duplexes against ALDH1A1 using a retroviral vector. We supplied the retroviral vector pLXSN, described before [12], as well as the siRNA AD1 sequence to GenScript (Scotch Plains, NJ) for the construction of expression vector containing the short hairpin RNA under the control of the U6 promoter targeting ALDH1A1. The following sequence was inserted: CTCGAGCGGACAATGCTGTTGAATTT CCACACCAAATTCAACAGCATTGTCCTTTTTTC CAAGGATCC. The same vector without the siRNA sequence was also constructed to be used as a control. The final constructs were sequencing verified by GenScript before delivery to us. A549 cells were transfected with either vector using electroporation (Bio-Rad Gene Pulser; Bio-Rad Laboratories, Richmond, CA) as described before [14]. Successfully transfected cells were selected using G-418 (up to 1 mg/ml) and used for experiments described below. These experiments include measurement of ALDH-1A1 by activity assay and Western blot, as well as the effect of ATRA and 4-HC treatment.

ATRA and 4-HC treatment

ATRA was dissolved in ethanol and added at 2 μM to cells with or without siRNA expression 2–3 days before

cell harvest. Control cells received equal amount of ethanol. Cell count and viability were determined before any further use of the cells in the different assays. Viability was determined by the trypan blue exclusion criteria. Cells were harvested using 250 µl lysing buffer containing 50 mM Tris pH 8.0, 25 mM EDTA, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl-flouride (PMSF), and 0.1% sarcosyl. After centrifugation at 10,000 rpm at 4°C for 10 min, cell lysate supernatant was collected and used to determine ALDH protein and activity, as described below. The protein concentration was determined using the BioRad protein assay kit with a bovine serum albumin (BSA) as a standard (BioRad, Hercules, CA).

For the 4-HC treatment, cells were trypsynized, washed, counted and aliquoted (10^5 cells/2 ml) into 15 ml tubes, as previously published [14, 15]. After about 2 h in the incubator, cells were treated with freshly prepared 4-HC for 30 min at 37°C. The cells were then washed twice with chilled culture medium, re-suspended in culture medium, and left in the incubator overnight for next day cytotoxicity assay. These A549 cells continue to proliferate in suspension culture (1.5±0.3 fold increase over 24 h) although slower than adhered cells (2.4±0.5 fold increase over 24 h).

Cytotoxicity assay

Cytotoxicity Detection Kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany) and used to measure the percentage cytotoxicity by determining LDH activity in the supernatants of treated cells as described before [15]. We followed the manufacturer's provided protocol and untreated control cells for each experiment group were incubated similarly to those treated with 4-HC and used to measure the high control (from the cell pellet) and the low control (from the supernatant of the cell pellet). The optimal cell concentration per group and the optimal drug concentration were first determined by preliminary experiments in order to find the cell concentration that will give the maximal difference between the low and high controls.

Western blot analysis

Cell lysates were used for Western blot analysis to demonstrate changes in protein levels of ALDH1A1 and ALDH3A1. Lysates from each experimental group were size separated in parallel on two 12% denaturing SDS-polyacrylamide gels (Biorad, Hercules, CA), electrotransferred onto nitrocellulose membranes, blocked with 5% milk in TBS, and probed as described before [14, 15], using chicken anti human ALDH1A1 and ALDH3A1 polyclonal antibodies provided generously by Dr. L. Sreerama (St Cloud University, Minneapolis, MN) and Dr. NE Sladek (University of Minnesota, Minneapolis, MN). The specificity of these antibodies has been documented by Dr. Sladek's group [6, 22]. Blots were incubated with chicken anti-human ALDH1A1 and ALDH3A1 primary antibodies at 1:200 or 1:300 dilution, respectively, for 1 h at room temperature. After washing, the secondary antibody (horseradish peroxidase-labeled rabbit anti-chicken antibody; Sigma Chemical Co., St Louis, MO) was used at 1:4000 dilution for 1 h. Chemiluminscence method (SuperSignal, Pierce, Rockford, IL) was used for the final visualization of the protein bands on X-ray film (Super Rx, Fuji Photo Film, Tokyo, Japan).

After washing and blocking, the same blots were labeled again for visualization of actin as a loading control using anti-actin antibody (Oncogene Research Products, Cambridge, MA). To quantitate the protein bands, the X-ray films were scanned using the ScanJet (Hewlett Packard), and integrated density was measured using ScionImage computer program (Scion Corporation). Relative normalized units were obtained by dividing protein level of ALDH by the actin level. The relative normalized units obtained from the ALDH and actin proteins of untreated cells in each blot served as the 100% control and the same ratio from experimental samples was used to calculate the percent change in ALDH protein.

ALDH activity assay

The cell lysates used for Western blot analysis, were also freshly used to measure ALDH enzyme activity using the spectrophotometric assay as described before [15]. Briefly, the aliquots of 600 µl lysing buffer were incubated at 37°C in Beckman DLC 64 spectrophotometer cuvettes with the addition of cell lysate, 5 mM NAD^+ and 5 mM propionaldehyde as a substrate. The change in absorbance at 340 nm was measured in three replicates over 5 min. A control reaction in which the substrate was not added monitored the endogenous rate of NAD^+ reduction. The ALDH activity was expressed in nmoles/10^7 cells min or nmoles/mg protein min.

Because propinaldehyde is an excellent substrate for ALDH class-2 (ALDH2), we performed semiquantitative RT-PCR using specific primers (Table 1) for ALDH1A1, ALDH3A1, and GAPDH as a house keeping gene control. The sequence of the primers for GAPDH and ALDH3A1 have been previously published [23, 24]. Total RNA was extracted using RNeasy Mini Kit (Quiagen), according to the protocol provided by the supplier, from wild type A549 cells. For the RT reaction, oligo (dT) was used to generate first strand for the antisense transcripts according to the manual Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The RT reaction was carried out separately, followed by PCR in which the specific primers in Table 1 were used. The program for PCR was previously published for the ALDH3A1 synthesis [24].

Statistical analysis

Graphs were prepared using Microsoft Office Excel or GraphPad Prizm (GraphPad Software, San Diego, CA).

Table 1 Primer Pairs Used in RT-PCR

Gene	Forward primer	Reverse primer	Product (bp)
ALDH1A1	GCATTGTGTTAGCTGATGCCG	AGAGAACACTGTGGGCTGGACA	342
ALDH3A1	ACTGGGCGTGGTCCTCGTCATTGG	GTGAGGATGGTGGGGGCTATGTAG	653
ALDH2	CCCGCCGTGGGCCACGCCTGA	GTTCTTCTGAGGCACTTTGAC	373
GAPDH	GAAGATGGTGATGGGATTTC	GAAGGTGAAGGTCGGAGTC	229

Statistical significance of the difference between the experimental groups was calculated using student t-test for two means. A two-tailed P value of <0.05 was considered significant.

Results

The shRNA cassette is effectively taken up by the A549 cells

In order to verify that the A549 cells are being transfected efficiently by siRNA, we transfected cells using the cassette containing the 1A1a sequence incorporated in a hairpin form under the human U6 promoter and labeled with 5′-CY3. Figure 1 shows strong epifluorescence observed under the microscope indicating significant transfection efficiency. Although this experiment was not intended to quantify the transfection efficiency, it was estimated to be between 30 and 50% in this experiment on two occasions.

The siRNA duplexes decrease protein expression and enzyme activity

Overall, and based on multiple experiments, we find that the siRNAs, designed to silence the ALDH1A1, decreases this enzyme activity to as low as 56±22%, and the protein level to a lowest level of 32±11%. The effect was specific, because all siRNAs designed for the ALDH1A1 did not have any significant effect on ALDH3A1 isozyme (see Summary Table 2).

The same can be said about the effects of siRNAs designed to silence the ALDH3A1 with the result of a decrease in this enzyme activity to as low as 54±16% and the protein level to lowest level of 41±23%. Again, the effect was specific in most but not all siRNAs used, as shown in summary Table 2.

The two duplexes for ALDH1A1 available from Dharmacon were pre-tested in A549 cell line at 48 and 72 h, and also at different concentrations (20–200 nM). Based on their effect on protein levels of ALDH1A1, we used the 200 nM concentration for subsequent experiments. However, even with high concentration, the AD1 and AD2 duplexes decreased the ALDH-1A1 protein to a 49 and 70% of control, respectively, at 72 h after transfection (data not shown). Because of the longer half life of ALDH1A1, we followed the effects of both of them for 6 and 9 days after transfection. The biggest effect

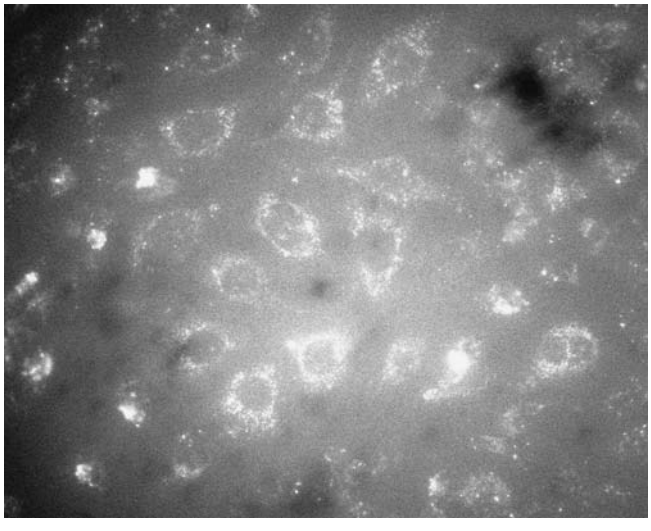

Fig. 1 Transfection efficiency of A549 cells using siRNA cassette containing 1A1a sequence under human U6 promoter and labeled with fluorescent dye 5′-Cy3. The number of fluorescent cells was estimated using a fluorescence microscope

Table 2 Summary Table. The following is a summary of the effects of all siRNA duplexes used (at optimal transfection conditions) on the enzyme activity as well as protein levels of both ALDH-1A1 and ALDH-3 A1

siRNA	Remaining activity[a]	Protein (Western blot)[b]	
		ALDH-1A1	ALDH-3A1
ALDH-1A1			
1A1a	66±5	67±4	98±8
1A1b	81±6	48±15	116±2
AD1	56±22	32±11	132±39
AD2	56	44	100
pL(AD1)SN	47±9	59±15	90±8
ALDH-3A1			
3A1A	76±1	144±27	89±12
3A1B	73±14	100	94
Smart Pool	ND	90	35
D1	54±16	104±35	41±23
D2	ND	94	40±10
D3	ND	42	56±10
D4	ND	25	58±48
Combinations			
D1 + AD1	37±14	15±11	99±21
D1 + pL(AD1)SN	33±9	50	67

ND Not done

[a] Percent remaining activity, with the 100% control for siRNA duplexes being oligofectamine only (Olf) group, and for pL(AD1)SN being pLXSN vector only cells

[b] Protein values are expressed as % control (cells exposed to oligofectamine only). Values reflect either mean ± standard deviation from ≥2 experiments or only one experiment value

occurred 6 days after transfection with AD1 duplex resulting in a decrease in ALDH1A1 protein level to 23% and no effect on ALDH3A1 (data not shown). On the other hand, AD2 was less effective with a maximum drop in ALDH1A1 protein of 44% (Table 2). Figure 2a shows a Western blot from one experiment in which AD1 effect is compared to the pool of non-specific duplexes, and it reveals a specific decrease in ALDH1A1 protein at 2 and 6 days (lanes 3 and 7) after transfection with AD1. The X-ray films from the Western blot were scanned and integrated density was measured using ScionImage computer program. Relative normalized units were obtained by dividing protein level of ALDH1A1 or ALDH3A1 by the actin level. Units obtained from the oligofectamine only cells (Olf, lanes 2 and 6) served as the 100% control, while units obtained from AD1 (lanes 3 and 7), untreated cells (lanes 1 and 4) or Pool (lanes 4 and 8) were expressed as a percentage of that and then blotted as shown in Fig. 2b for ALDH1A1 and Fig. 2c for ALDH3A1. Black bars indicate results from day 2, and white bars indicate results from day 6 of cell culture. The results show 41 and 28% remaining ALDH1A1 protein on days 2 and 6, respectively, after transfection with AD1. Decrease in ALDH3A1 protein was seen only at 6 days after transfection with AD1 and non-specific siRNA pool (Fig. 2c). Based on these results we chose the AD1 duplex for insertion into pLXSN retroviral vector in order to achieve long-term expression.

The SmartPool siRNA for ALDH3A1

The SmartPool for the ALDH3A1, consisting of four oligonucleotide duplexes transfected into A549 cells, caused the decrease of the ALDH3A1 protein level to as low as 35% after 72 h, without significant decrease in ALDH1A1 protein (Table 2). In contrast to the peak effect on ALDH1A1 down-regulation seen at day 6 (Fig. 2), the protein level of ALDH3A1 was already going up at day 6 (data not shown).

Four SmartPool components for the ALDH3A1 (D1-D4) were pre-tested separately at 48 and 72 h after transfection, and also at different concentrations 20–200 nM. The most effective and specific was the D1 duplex which we used for subsequent experiments. A549 cells transfected with 200 nM of D1 showed significant decrease in ALDH3A1 protein to as low as 52 and 23% of control after 48 and 72 h, respectively. The overall mean decrease in ALDH3A1 protein 72 h after transfection with D1 was 41% (Table 2) with no effect on ALDH1A1 protein.

Single siRNA or in Combination with Each Other As Well As with ATRA ALDH1A1 activity was reduced to about 50% of control by the permanent expression of AD1 duplex in A549 cells using the pLXSN retroviral vector which is also reflected in a proportional decrease in ALDH-1A1 protein without significant effect on ALDH-3A1 protein level as shown in Western blot (Fig. 3) and in summary Table 2. The overall effect of

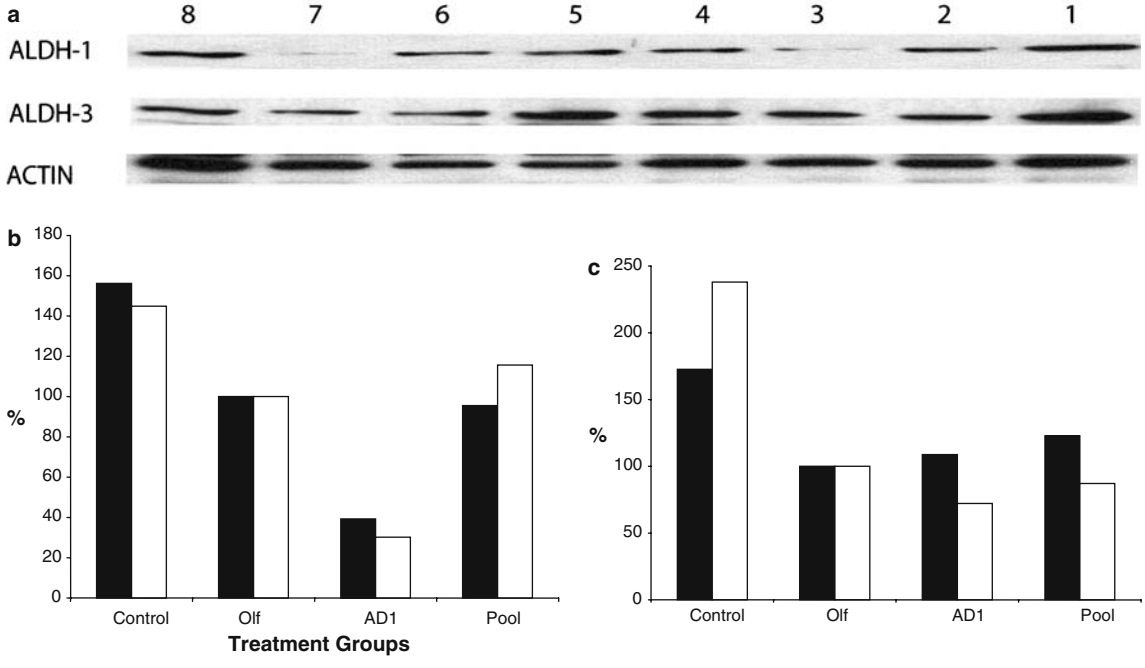

Fig. 2 Knockdown of ALDH1A1 by AD1 duplex in comparison to non-specific pool of siRNA duplexes (Pool). **a** Western blot analysis of A549 cells transfected with AD1 (lanes 3 and 7), Pool (lanes 4 and 8), versus untreated wildtype cells (control, lanes 1 and 5) and cells treated with oligofectamine only (Olf, lanes 2 and 6) and then harvested on day 2 (lanes 1–4) and 6 (lanes 5–8) of culture. Blot was probed with antibodies to ALDH1A1, ALDH3A1 and actin. **b** X-ray films of Western blot were scanned and integrated density was measured using ScionImage computer program. Relative normalized units were obtained by dividing protein level of ALDH1A1 by the actin level. Units obtained from Olf group served as the 100% control while units obtained from AD1, Pool, or untreated control groups were expressed as a percentage of that. *Black bars* indicate results were obtained from day 2 while *white bars* indicate results were obtained from day 6 after siRNA transfection. **c** Similar calculations were performed for ALDH3A1 like in **b**

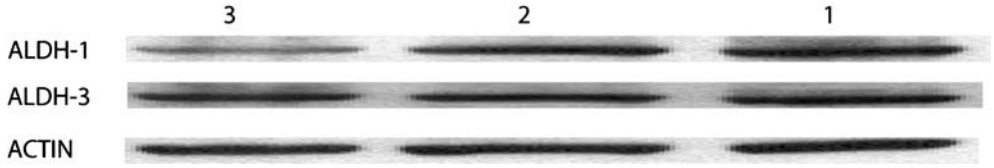

Fig. 3 RNAi-mediated knockdown of ALDH1A1 using a retroviral vector. Western blot analysis of A549 cells that were transfected with either empty pLXSN vector or pL(AD1)SN, selected in 1 mg/ml G418, and then harvested for protein extraction. Blots were probed for ALDH1A1, ALDH3A1 and actin. The results show specific decrease in ALDH1A1 protein only in the pL(AD1)SN (lane 3) group in comparison to pLXSN (lane 2) and wild type cells (lane 1)

AD1 expression by retroviral vector on ALDH1A1 was similar to that seen by the use of AD1 duplex. Figures 4 and 5 are illustrating both the inhibitory effect of the siRNA only (white bars) as well as the effect of adding ATRA to the siRNA (checked bars) on ALDH enzyme activity. Figure 4 shows significant decrease in ALDH activity ($P=0.0066$) when compared to oligofectamine-treated cells (Olf) or cells treated with non-specific pool of siRNAs (Pool). It also shows that the inhibitory effects of AD1 and D1 (siRNA for ALDH3A1) on enzyme activity are similar. Figure 5 shows that stably transfected cells with pL(AD1)SN display significantly reduced enzyme activity ($P<0.0001$) in comparison to cells stably transfected with empty pLXSN vector or wild type cells (WT). These results from Figs. 4 and 5 reveal similar inhibitory effects on ALDH activity by AD1 duplex or AD1 expressed via retroviral vector. Because of these results, we elected not to pursue the same approach of retroviral-mediated expression of siRNA for ALDH3A1.

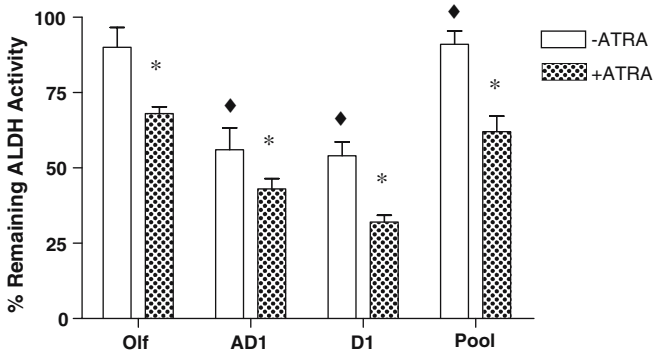

Fig. 4 The effect of AD1 or D1 siRNA duplexes ± ATRA on ALDH enzyme activity. A549 cells were exposed to 200 nM of AD1 or D1 for 72 h with 2 μM of ATRA (*checked bars*) or without (*white bars*), then harvested, counted and enzyme activity was measured using spectrophotometry. The control groups included untreated cells which also served as the 100% control for the activity (not shown), cells treated with oligofectamine (Olf) which is the transfection vehicle, and cells treated with non-specific pool of siRNA duplexes (Pool) (Dharmacon). The results represent the mean ± SEM of the percent remaining activity compared to the untreated control cells from three experiments. In comparison to control groups (Olf and Pool), significant reduction of activity was observed in the AD1 ($^{\blacklozenge}P=0.0066$) and D1 treated cells ($^{\blacklozenge}P=0.0018$) in the absence of ATRA. The addition of ATRA resulted in significant reduction of activity in all experimental groups ($^{*}P\leq0.036$)

Based on the results above, we chose AD1 duplex for ALDH1A1 and D1 duplex for ALDH3A1 to test their effect on overall ALDH enzymatic activity in A549 cancer cells. However, the results with using the D1 and AD1 duplexes in combination were variable from one experiment to another and showed no consistent effect on ALDH3A1 protein levels (as shown in Table 2) and had no additive effect on the 4-HC toxicity (shown in Table 3). Since we did not have a good explanation for this phenomenon, we used D1 duplex in the A549 cells stably transfected with either pLXSN or pL(AD1)SN in order to achieve consistent and reproducible results. Summary Table 2 shows that D1 reduced ALDH activity to 54±16% in cells already expressing pLXSN vector (control cells), while cells transfected with pL(AD1)SN have reduced ALDH activity to 47±9% in comparison to pLXSN only cells and the activity is further reduced by 100 nM D1 to as low as 33±9%. Figure 6 shows the effect of combining D1 and pL(AD1)SN on actual enzyme activity in comparison to two controls of combining pL(AD1)SN with non-specific siRNA pool (Pool) or the transfection vehicle oligofectamine (Olf). Thus, an expected added inhibitory effect on ALDH activity is clearly seen when using combination siRNA duplexes against both enzymes but full additive effects was never

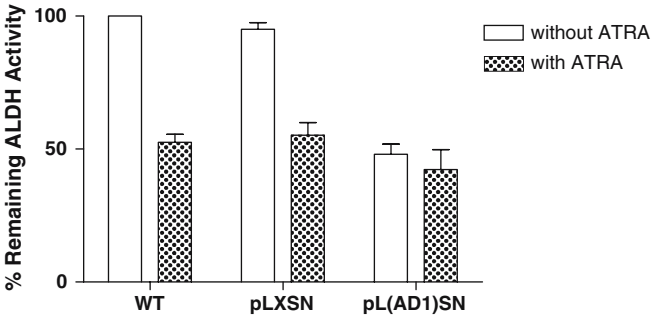

Fig. 5 ATRA effect on ALDH enzyme activity when combined with RNAi-mediated knockdown of ALDH1A1. Stably transfected A549 cells with pLXSN only, pL(AD1)SN or wild type (WT) cells were treated with 2 μM ATRA for 72 h, harvested, lysed, and ALDH activity measured using spectrophotometry. *Bars* indicate means ± SEM of remaining ALDH activity from seven similar experiments. Significant reduction in ALDH activity was seen in pL(AD1)SN cells compared to untreated WT or pLXSN cells (*white bars*, $P<0.0001$). ATRA treatment (*checked bars*) resulted in significant reduction in ALDH activity in WT and pLXSN cells ($P<0.0001$), but not when added to pL(AD1)SN ($P=0.254$)

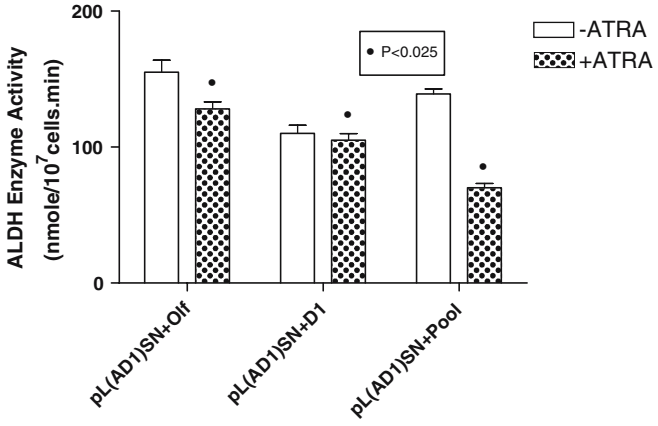

Fig. 6 ATRA effect on ALDH enzyme activity when combined with RNAi-mediated knockdown of both ALDH1A1 and ALDH3A1. Stably transfected A549 cells with pL(AD1)SN were incubated with oligofectamine (Olf), 200 nM D1, or non-specific pool (Pool) with or without 2 μM ATRA. After 72 h, cells were harvested, washed, lysed, and enzyme activity measured by spectrophotometry. *Bars* indicate means ± SEM from two experiments (total of six measurements) of actual ALDH activity measurements (nmole/10^7 cells min). Significant reduction in ALDH activity was seen with the addition of D1, but not Pool, to pL(AD1)SN transfected cells (*white bars*). ATRA addition (*checked bars*) resulted in significant further decrease in ALDH activity when added to all groups except the group with cells exposed to siRNA mediated inhibition of both enzymes

seen and never lead to complete or near complete inhibition of activity. The results of RT-PCR show that ALDH2 is expressed in A549 cells at low level in comparison to ALDH1A1 and ALDH3A1 (Fig. 7), and therefore the remaining ALDH activity could in part be attributed to ALDH2.

In order to test the effect of ATRA in combination with specific siRNA duplexes, 2 μM was added to AD1 duplex, D1, and pL(AD1)SN for 72 h before harvesting the cells. ATRA significantly decreased ALDH activity in all control cells, but had less significant effect when combined with either AD1, pL(AD1)SN or D1 alone, but the least inhibitory effect on enzyme activity was seen when combined with siRNAs against both enzymes (See

Table 3 Effect of siRNA and ATRA on A549 cells sensitivity to 80 μg/ml 4-HC

Groups	% increase in LDH release		
	ATRA	4-HC	ATRA+4-HC
Olf (control)	0	25	38 (51%)
Olf + AD1	8	46 (84%)	51 (102%)
Olf + AD1 + D1	3	39 (56%)	57 (128%)
Olf + D1	7	40 (60%)	53 (112%)

LDH cytotoxicity assay was used to measure the toxicity of 4-HC in the different experimental groups. Cells exposed to Olf only were considered the control cells and the changes in toxicity were calculated according to that after exposure to ATRA, 4-HC, or both. The values are taken from one of two experiments and represent % release of LDH reflecting the 4-HC toxicity. The values in brackets represent % increase in toxicity over control
AD1 siRNA duplex to ALDH1A1; *D1* siRNA duplex to ALDH3A1; *Olf* oligofectamine, the transfection vehicle

Figs. 4, 5, 6). When assessing the results of each experiment separately, then there is a clearer added inhibitory effect of ATRA on ALDH activity and the median additional decrease in ALDH activity from several experiments (*n*) was as follows: with D1- 31% ($n=3$, range 24–38); AD1- 21% ($n=7$, range 15–50); pL(AD1)SN- 13% ($n=8$, range 2–33); pL(AD1)SN+D1- 5% ($n=5$, range 0–33), while with the control (pL(AD1)SN + non-specific Pool)- 42% ($n=4$, range 17–56). We conclude that the effect of ATRA is much less noticeable when added to cells treated with combination siRNA for both enzymes in comparison to cells treated with single siRNA or the control cells.

4-HC toxicity

The same batch of siRNA-transfected A549 cells that were used to check the ALDH protein levels was used to test the 4-HC toxicity. The results of one of three similar experiments are shown in Table 3. A549 transfected with AD1 displayed an increase in 4-HC (80 μg/ml) toxicity by as much as 84% when compared to the control (Olf + 4-HC treated) cells. Similarly, A549 transfected with D1 had an increase in 4-HC toxicity by as much as

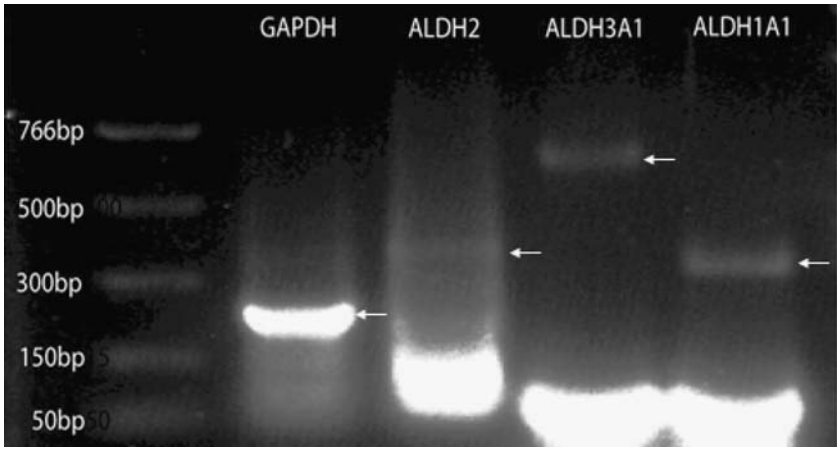

Fig. 7 A549 cells were analyzed for the expression of ALDH2, ALDH3A1, and ALDH1A1 using semi quantitative RT-PCR. GAPDH house keeping gene was used as a control. A representative ethidium bromide stained 2% agarose gel shows that ALDH2 mRNA (product size of 373 bp) is detectable at very low levels in comparison to ALDH1A1 and ALDH3A1. PCR Marker (New England BioLabs) was used in the first lane

60% when compared to the control, however, no further increase in 4-HC toxicity was seen when combining AD1 + D1 (100 nM each). On the other hand, a significant increase in 4-HC toxicity ($P < 0.01$) was seen when D1 (100 nM) was combined with pL(AD1)SN (Fig. 8, white bar labeled D1 versus white bar labeled control). These results match the inhibitory effect of siRNA duplexes on ALDH1A1 and ALDH3A1 and demonstrate the separate role for each enzyme in promoting resistance to 4-HC.

Incubation of same siRNA-transfected A549 cells with 2 μM ATRA for 3 days before treatment with 80 μg/ml 4-HC resulted in further increase in toxicity of up to 102% (with 200 nM AD1) and 112% (with 200 nM D1) in 4-HC toxicity when compared to the control cells (Table 3). The ATRA itself increased cytotoxicity by 52%. The percent release of LDH from the cells before any 4-HC treatment was not different among the experimental groups indicating no significant toxicity caused by the ATRA itself and only minimal toxicity when ATRA added to the siRNA duplexes (Table 3, ATRA column). Considering the insignificant effects of ATRA on ALDH activity when added to pL(AD1)SN+D1 (Fig. 6), and the results showing further and significant increase in 4-HC toxicity (checked bars, Fig. 8) under the same conditions, we believe that ATRA is also increasing 4-HC toxicity through additional mechanism(s) other than its effect on ALDH.

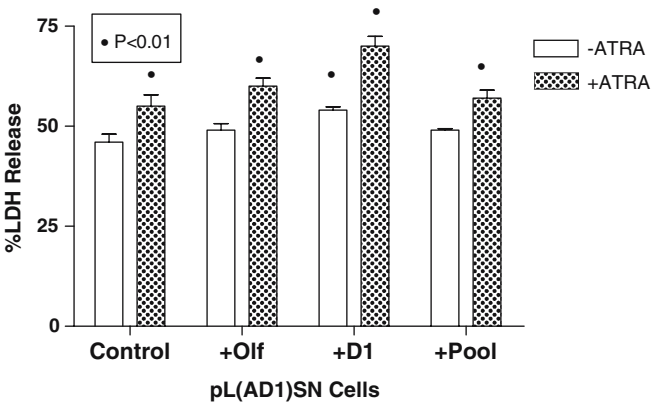

Fig. 8 The effect of RNAi-mediated knockdown of both ALDH1A1 and ALDH3A1 on 4-HC cell sensitivity. Stably transfected A549 cells with pL(AD1)SN (control) were incubated in the presence of oligofectamine only (Olf), 100 nM D1, or 200 nM non-specific Pool (Pool), and with (*white bars*) or without 2 μM ATRA (*checked bars*). After 72 h incubation, cells were harvested, washed, and 10^5 viable cells/2 ml culture medium were treated with 80 μg/ml 4-HC for 30 min at 37°C. After washing twice in chilled medium, cells were incubated overnight for next day assay for LDH release using the Cytotoxicity Detection kit (Roche Molecular Biochemicals). Bars indicate means ± SEM from two experiments (total of 6 measurements) of percent LDH release which reflects 4-HC cytotoxicity. For comparison, cells transfected with pLXSN only and treated with 80 μg/ml 4-HC show 33±3% of cytotoxicity

Discussion

ALDH1A1 and ALDH3A1 have been studied extensively for their role in drug resistance against oxazaphosphorines, specifically cyclophosphamide and its active derivatives. The expression of these enzymes can vary from one cancer to the other [25], and therefore it has become important for us to determine the contribution of each one to such resistance. We have previously published on the use of RNA antisense as a mean to downregulate each one of these enzymes and thus determine their role in the reversal of resistance to 4-HC [14]. However, the low specificity and efficacy using the RNA antisense approach prevented us from reaching conclusions in regards to the role of each enzyme. In the present study, we use siRNA duplexes as well as retroviral vector mediated expression of siRNA in order to "knock down" the expression of these enzymes in A549 lung carcinoma cells, which are known to have very high levels of both enzymes [15]. Overall, we found that siRNA duplexes are effective and more specific than RNA antisense in decreasing ALDH1A1 and ALDH3A1 activity and/or protein levels.

The siRNA effect on enzyme activity and protein was seen as early as 48 h after transfection and lasted as long as 6 days. The maximum inhibitory effect seen on the protein level of either ALDH isozyme was approximately 80% reduction, and the maximum drop in ALDH3A1 protein was seen earlier than that of ALDH1A1 after siRNA transfection.

The use of appropriate siRNA not only decreased the ALDH activity and/or protein expression, but also increased 4-HC-mediated cytotoxicity. The inhibition of each enzyme separately resulted in a similar increase in 4-HC toxicity, thus indicating no difference in the contribution of each enzyme to resistance against the active metabolite of cyclophosphamide. The clinical implication of these findings is that both enzymes will need to be inhibited when attempting to effectively overcome cyclophosphamide resistance in cancer cells. The use of a combination of siRNA duplexes proved to be problematic in terms of reproducibility with variability of inhibitory effects on ALDH activity and lack of significant decrease in ALDH3A1 protein levels. The addition of ALDH3A1 siRNA dupex D1 to cells expressing retroviral vector containing AD1 siRNA for ALDH1A1, resulted in a more consistent effect on ALDH activity. Despite that, the ALDH activity was never "knocked down" to zero. Thus, the use of one siRNA sequence that will affect both enzymes or the use of different vector systems such as lenti virus may be needed to improve upon these results. We have also shown that ALDH2 is expressed at very low level in A549 cells and may contribute to the remaining enzyme activity. However, ALDH2 has not been a significant player in the resistance to oxazaphosphorines and therefore it should not affect the main conclusions in this study.

The use of siRNA duplexes against ALDH1A1 and ALDH3A1 has been reported before in investigating their role in the defense against oxidative stress of rat

and human lens epithelial cells [26]. Interestingly, the human sequences used were different than the ones we used in the current study. The effect was measured differently using RT-PCR and measurement of the end effect on HNE-detoxifying capacity, and therefore, it is not possible to compare our results to those published by Choudhary et al. However, it is clear that our present study contains the most extensive experience in RNAi-mediated knockdown of ALDH1A1 and ALDH3A1. Although enzyme inhibitors are available against certain ALDH isozymes and have been used to study ALDH, the advantage of using state of the art technology, such as RNAi approach, to achieve specific inhibition of some of these isozymes should allow dissecting out the overlapping biologic functions that exist among some of these isozymes. Thus, we hope that this experience will be helpful for other investigators interested in the different biological activities of these enzymes whether it is in embryogenesis or oxidative stress response [27, 28].

Our previous studies showed that retinoids-induced reduction in ALDH-1A1 and ALDH3A1 resulted in increased acetaldehyde and 4-HC toxicity [15]. Therefore in this study we also used ATRA alone or in combination with siRNA. Once again, we found that ATRA alone at a concentration of 2 μM was not toxic to the cells, but significantly reduced ALDH activity and increased 4-HC cytotoxicity. Furthermore, the addition of ATRA to cells transfected with siRNA against either enzyme resulted in a further reduction in the median ALDH activity and significantly higher 4-HC toxicity. On the other hand, the effect of ATRA on enzyme activity when combined with siRNAs against both enzymes [pL(AD1)SN+D1] was minimal, yet it resulted in a small but significant increase in 4-HC toxicity. These results suggest that ATRA has an enhancing effect on the sensitivity to 4-HC through a different pathway other than the reduction in ALDH enzyme activity and protein. Such an effect has been suggested by previous studies [29–33] in which ATRA increased the toxicity of other chemotherapeutic agents not metabolized by ALDH. Moreover, Pappa et al. [28] reported recently that ALDH3A1 transfected corneal epithelial cells exhibited increased resistance to the cytotoxic effects of mitomycin C and VP-16 through ALDH3A1 mediated protection against oxidative damage. These studies suggest that at least some of the other reported effects of ATRA on increased drug toxicity may be mediated through its effect on ALDH3A1 protein. In order to verify that possibility further, we need to achieve close to 100% reduction in ALDH activity and possibly use cell lines that express ALDH3A1 only.

In summary, our observations in regards to the combination of RNAi, ATRA and cyclophosphamide may have future clinical implications in the treatment of lung cancer, although significant challenges will still need to be overcome in the in vivo application of RNAi, which are similar to those known from numerous gene therapy studies [34].

On the other hand, our observations could serve as the basis for studying and revealing the regulatory mechanisms involved in determining the cellular expression and importance of these enzymes in the evolution of cancer.

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