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TITLE: **Retinoic acid downregulates aldehyde dehydrogenase and increases cytotoxicity of 4-hydroperoxycyclophosphamide and acetaldehyde[1]**

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Nonstandard Abbreviations used:

aldehyde dehydrogenases (ALDH)

4-hyroperoxycyclophosphamide (4-HC)

all-trans retinoic acid (ATRA)

phenylketophosphamide (PKP)

aldehyde dehydrogenase class-1A1 (ALDH-1A1)

aldehyde dehydrogenase class-3A1 (ALDH-3A1)

non-small cell lung cancer (NSCLC)

American Type Culture Collection (ATCC, Rockville, MD)

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ABSTRACT

Multiple prior studies have identified aldehyde dehydrogenases (ALDH) able to oxidize retinal to retinoic acid. In this study, we test the hypothesis that the accumulation of intracellular retinoic acid may lead to the suppression of ALDH expression and thus increase cytotoxicity to 4-hydroperoxycyclophosphamide (4-HC) *in vitro*. Mainly A549, but also other lung cancer cell lines, were used in our experiments with the former having high levels of two ALDH isozymes expressed. Dose response and time course were performed incubating the cells with all-trans retinoic acid (ATRA) as well as other commercially available retinoids. The results show that incubation of A549 cells with any of the retinoids at pharmacologic doses for $\geq$48 hr results in significant decrease in ALDH-1A1 and ALDH-3A1 enzyme activity and protein levels, but not the corresponding mRNAs. Such decrease in ALDH activity was seen in all cell lines tested and results in a significant increase in toxicity of 4-HC and acetaldehyde, both are substrates for the enzymes. Prior incubation with ATRA also results in increased cytotoxicity, although to a lesser degree, of phenylketophosphamide (PKP) and melphalan, neither is substrate for ALDH. These results suggest a posttranslational mechanism through which retinoids decrease both ALDH expression, which results in increased cytotoxicity of 4-HC and acetaldehyde, although other previously described effects of these retinoids may be contributing to the slight increase in cytotoxicity seen with other chemotherapy agents. These results may have clinical implications in regards to the use of retinoids in lung cancer prevention and treatment.

INTRODUCTION

Aldehyde dehydrogenases are a group of enzymes catalyzing the conversion of a broad range of aldehydes to the corresponding acid via a NAD^+ -dependent irreversible reaction. Two of these enzymes, cytosolic aldehyde dehydrogenase class-1A1 (ALDH-1A1) and class-3A1 (ALDH-3A1) have been found to be responsible for drug resistance in various tumor types against the antineoplastic drugs collectively known as oxazaphosphorines which include cyclophosphamide and its active metabolites (Hilton, 1984; Manthey et al., 1990; Sreerama and Sladek, 1993; von Eitzen et al., 1994; Yoshida et al., 1998). Cyclophosphamide is a prodrug requiring cytochrome P-450 hydroxylation for activation. Prior to release of the active alkylating metabolite phosphoramide mustard, cyclophosphamide passes through an aldehyde intermediate, aldophosphamide. ALDH oxidizes aldophosphamide to the inactive metabolite carboxyphosphamide (Manthey et al., 1990). Several inhibitors of ALDH activity have been used to demonstrate the reversal of this drug resistance mechanism. We and others (Bunting et al., 1994; Bunting and Townsend, 1996; Magni et al., 1996; Moreb et al., 1996; Moreb et al., 1998) have successfully shown that over expression of ALDH-1A1 or ALDH-3A1 in cell lines and in normal hematopoietic progenitors results in significant increase in resistance to the active metabolites of cyclophosphamide in vitro. Furthermore, we have shown that downregulation of ALDH-1A1 (Moreb et al., 2000) and ALDH-3A1 (unpublished data) using antisense RNA result in increased sensitivity of tumor cells to 4-

hydroperoxycyclophosphamide (4-HC), an active derivative of cyclophosphamide.

Multiple prior studies have identified several ALDHs able to oxidize retinal to retinoic acid, a modulator of gene expression and cell differentiation and maintenance of numerous tissues (Chytil, 1996; Gudas et al., 1994; Means and Gudas, 1995). Human, mouse and rat ALDH-1A1 has been reported to have high activity for retinal oxidation (Bhat et al., 1995; Hsu et al., 1999; Lee et al., 1991; Yoshida et al., 1992). Thus, in this study we test the hypothesis that accumulation of intracellular retinoic acid may lead to the suppression of ALDH expression and thus increase 4-HC cytotoxicity in vitro. Our results suggest that retinoids indeed suppress levels of ALDH-1A1 and ALDH-3A1 protein and enzyme activity in physiologic and pharmacologic doses. The effects of retinoic acid on ALDH-1A1 and ALDH-3A1 provide new insight into the posttranslational regulation of the enzymes.

METHODS

CELL LINES

The A549 non-small cell lung cancer (NSCLC) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cells were grown in RPMI-1640 culture medium supplemented with 10 % fetal bovine serum and used when in the exponential log phase of their proliferation.

Other lung cancer cell lines were used including SW210.5 (small-cell lung cancer) and ADLC-5M2 (NSCLC) that were obtained from Dr. Gerold Bepler (now at H. Lee Moffitt Cancer Center, Tampa, FL) (Bepler et al., 1988; Bepler et al., 1989).

MATERIALS

All retinoids, all-trans RA (ATRA), 9-cis and 13-cis retinoic acids, were purchased from Sigma Chemical Co. (St. Louis, MO). NAD, propionaldehyde, acetaldehyde, and melphalan were also purchased from Sigma/Aldrich. All retinoids were dissolved in ethanol. Melphalan was dissolved in small amounts of ethanol and acetic acid and then diluted in culture medium to the appropriate concentration.

4-HC and phenylketophosphamide (PKP) were supplied generously by Dr. Michael Colvin, Duke University, Durham, NC. 4-HC was dissolved in culture medium for 1 μg/μl solution. PKP was dissolved in DMSO and then diluted in culture medium for 1 μg/μl final solution.

Chicken anti human ALDH-1A1 and ALDH-3A1 polyclonal antibodies were 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 (Sladek et al., 2002; Sreerama and Sladek, 1993).

RA INCUBATIONS

ATRA was the main retinoic acid used throughout the experiments. The other retinoic acids were used for comparison with ATRA. A dose response effect on ALDH enzyme activity was performed using A549 cells after 72 hr incubation with a range of ATRA concentrations of 10^{-10}-10^{-5} M.

A time course was also performed using A549 cells and fixed 1µM ATRA concentration. ALDH enzyme activity was measured after 12 hr of ATRA incubation and every 24 hr up to 8 days. The starting cell concentration for A549 cells was either $0.5x10^6$ (up to 72 hr incubations) or $0.1x10^5$ cells (for longer incubations) in 15 ml RPMI-1640 in 75cm^2 flask. 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. Since ATRA is dissolved in 100% ethanol, control cultures received the same amount of ethanol as the ATRA-treated cultures in all the experiments.

MEASUREMENT OF ALDH ACTIVITY

Measurement of the human ALDH activity was performed as described before (Moreb et al., 2000). Briefly, cells were lysed in 0.5-1 ml buffer containing 50 mM Tris pH 8, 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, supernatant was collected and used to determine ALDH activity. The protein concentration was determined using the BioRad protein assay kit with a bovine serum albumin (BSA) standard (BioRad, Hercules, CA).

Aliquots of 600µl incubated at 37°C using Beckman DLC 64 cuvette spectrophotometer with the addition of protein extract, 5 mM NAD^+, and 5 mM propionaldehyde (as substrate) were used to measure the change in absorbance at 340 nm over 5 min. A control reaction in which propionaldehyde was not added monitored the endogenous rate of NAD^+ reduction.

WESTERN ANALYSIS

Western blot analysis was performed to demonstrate changes in protein levels of ALDH-1A1 and ALDH-3A1. The same protein extract used for ALDH enzyme activity (described above) was used for the Western blot analysis. Equal amounts of protein from each experimental group were size separated on a 10 % denaturing SDS-polyacrylamide gel, electrotransferred onto nitrocellulose membranes, and labeled as described before (Moreb et al., 2000). Chicken anti-human ALDH-1A1 and ALDH-3A1 primary antibodies were used at 1:200

dilution, and secondary antibody (horseradish peroxidase-labeled rabbit anti-chicken antibody; Sigma Chemical Co., St Louis, MO) was used at 1:6000 dilution. Chemiluminscence method (SuperSignal, Pierce, Rockford, IL) was used for the final visualization of the protein bands on X-ray film.

All blots were labeled again for visualization of actin as a loading control using anti-actin antibody (Oncogene Research Products, Cambridge, MA).

NORTHERN ANALYSIS

Northern analysis was used to detect changes in ALDH-1A1 and ALDH-3A1 mRNAs after incubation of cells with retinoic acid. Total RNA was extracted from A549 cells after different incubation times with ATRA using Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). Total RNA (10 μg/lane) was separated on 1.2% agarose formaldehyde gel and blotted onto a nylon membrane (MSI, Westboro, MA) by capillary transfer for 2 days. Detection of ALDH-1A1 and ALDH-3A1 was performed as described before (Moreb et al., 2000), using random-primer [^{32}P] dCTP-labeled ALDH-1A1 and ALDH-3A1 cDNAs.

IN VITRO DRUG TREATMENT ASSAY

After incubation with or without retinoic acid, cells were harvested. Following the determination of cell count and viability, A549 cells or ADLC-5M2 (1 x 10^5 cells/ 2 ml fresh culture medium) or SW 210.5 cells (4 x 10^5 cells/ 2 ml) were treated with 4-HC (40-80 μg/ml) for 30 min at 37°C, washed twice with

culture medium, then either plated for liquid colony assay (A549 cells only) or used for LDH cytotoxicity assay (see below) in order to determine the 4-HC cytotoxic effects.

In order to determine the specificity of retinoic acid effects on ALDH and sensitivity to 4-HC, we also treated A549 cells with drugs not metabolized by ALDH-1A1 or ALDH-3A1 such as PKP and melphalan. Again, cells with or without retinoic acid preincubation, were treated with the corresponding drug for 30 min (PKP) or 60 min (melphalan) at 37°C, washed twice, and used for the LDH cytotoxicity assay.

Because acetaldehyde is a carcinogen metabolized by ALDH, we examined the effect of ATRA preincubation on the acute toxicity of 24 hr exposure of A549 cells to acetaldehyde. In order to determine the toxic dose of acetaldehyde, we first performed a dose response with acetaldehyde using the LDH cytotoxicity assay.

CYTOTOXICITY ASSAYS

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. We followed the manufacturer's provided protocol. The optimal cell concentration per well 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.

A549 cells were also plated in liquid colony assay as described before (Moreb et al., 2000), in order to further measure the cytotoxic effects 4-HC with or without retinoic acid preincubation. After treatment, cells were resuspended in 4 ml RPMI + 10% FBS and plated in four 35 mm petri dishes for each experimental group. Colonies (>10 cells) adhered to the bottom of the plate were counted on day 4 using an inverted microscope. Untreated A549 cells were plated similarly at 200 cells/ml/dish. The toxicity of 4-HC was reflected by the amount of colonies killed and expressed as a percentage of total colonies recovered from equal numbers of untreated A549 cells.

STATISTICAL ANALYSIS

Statistical significance of the difference between experimental groups was calculated using student's paired *t* test for two means. A *P* value of < 0.05 was considered significant.

RESULTS

REGULATION OF ALDH EXPRESSION BY RETINOIC ACID

Because retinoic acid is the product of catalyzing aldehyde dehydrogenase, and in order to test a possible negative feedback effect of high levels of retinoic acid on ALDH expression, we incubated A549 cells with different ATRA concentrations as well as for different periods of time using 1 or 2 μM ATRA. The effect was measured by enzyme activity, Western and Northern analysis.

Figure 1 shows a dose response effect on ALDH enzyme activity using ATRA 10^{-10} - 10^{-5} M. Because a 10^{-6} M corresponds to concentration achievable by pharmacologic doses of ATRA, we used that concentration in subsequent experiments. A time course experiment revealed that ALDH enzyme activity decreases as early as 12 hr after the beginning of incubation with 1μM ATRA and continues as long as 8 days. The persistent decrease in ALDH activity is dependent on the addition of fresh ATRA every 48 hrs, otherwise a rebound of ALDH activity is seen about 72-96 hrs after the initial addition of single ATRA dose to the cell culture (data not shown). Also, in one experiment, we screened 13-cis and 9-cis retinoic acids for the longer incubation up to 8 day. Similar effects to that of ATRA on ALDH activity were seen by these two retinoic acids. Because of that, subsequent experiments were done with ATRA only. Table 1 and Figure 2 show a significant decrease in ALDH activity and protein, respectively. Both ALDH-1A1 and ALDH-3A1 proteins are similarly affected on different days of the incubation (Figure 2). Furthermore, the removal of ATRA

after only 3 hrs incubation will still result in a significant decrease in ALDH activity (20±3%) at 48 hrs of continued culture of A549 cells, indicating that the ATRA effect is mediated through retinoic acid receptors and its signal transduction pathways.

Northern analysis of RNA obtained from A549 cells after 72 hr incubation with 1μM ATRA showed no change in ALDH-1A1 or ALDH-3A1 mRNA levels (data not shown). These results suggest that ATRA effect on ALDH levels is through posttranslational regulatory mechanisms. During ATRA incubation, cell viability and count were determined at different time. Minimal effect on viability was noticed over 8 day incubation (Figure 3), however significant decrease in proliferation was noticed after 5 day incubation with ATRA as reflected by low cell counts in comparison to untreated cells (Figure 4).

The use of other commercially available retinoic acids such as 13-cis and 9-cis retinoic acids resulted in similar effects to that of ATRA on ALDH enzyme activity in A549 cells (Table 1). Only one screening experiment with 13-cis retinoic acid was performed over 3, 5, and 8 days, and the results in Table 1 show similar but less robust effect on ALDH enzyme activity to that seen with ATRA. Subsequently, and in view of similar results obtained from 72 hr incubations with the 3 different retinoic acids, we focused on performing studies using ATRA only.

In order to investigate whether the ATRA induced decrease in ALDH activity is limited to NSCLC A549 cell line, two other cell lines NSCLC cell line ADLC-5M2and SCLC cell line SW 210.5, were similarly incubated with ATRA for

72 hr and again significant inhibition ($P < 0.05$) of ALDH activity was seen (Figure 5), although the basal activity of ALDH in these cell lines was much lower than that in A549 cells.

RETINOIC ACID PREINCUBATION RESULTS IN INCREASED SENSITIVITY TO 4-HC

Preincubation of A549 cells with 1μM of ATRA, 13-cis or 9-cis retinoic acids for 3 days before treatment with 40-80μg/ml 4-HC (Figure 6, Panel A) results in significant increase in 4-HC toxicity (measured by LDH cytotoxicity assay) when compared to control cells. The % release of LDH from the cells in the different groups before any 4-HC treatment was not different among the four experimental groups indicating no significant toxicity caused by the retinoids themselves. Figure 6, Panel B, shows similar increase in 4-HC toxicity against A549 cells as measured by liquid colony assay. In order to verify whether such effect on 4-HC sensitivity correlates specifically to the decrease in ALDH enzyme activity, we treated A549 cells with different ATRA concentration for 72 hr and then treated with 80μg/ml of 4-HC. As expected, the results show that the increase in 4-HC cytotoxicity (as reflected by the relative amount of LDH release) corresponds to the degree of suppression of ALDH enzyme activity by the different ATRA dose level (Figure 7, Panels A&B). Furthermore, longer incubation (5 and 8 days) with ATRA or 13-cis retinoic acid does not generally result in more pronounced 4-HC toxicity (Table 2) but rather a plateau effect is seen. The LDH release at 0 μg/ml 4-HC was minimal and not significantly

different between the cells in the 3 treatment groups in Table 2 (similar to what is shown in Figure 6A), even after 8 days of incubation.

On the other hand, and in order to further investigate the specificity of the retinoic acids effect, we used other cytotoxic drugs that are not metabolized by aldehyde dehydrogenase such as PKP and melphalan. Table 3 shows that 72 hr ATRA preincubation results in increased cytotoxicity of PKP and melphalan, but to a lesser degree than that seen for 4-HC.

The other two cell lines, ADLC-5M2 and SW210.5, were similarly treated with ATRA and 4-HC. Toxicity was measured by LDH cytotoxicity assay. The results showed increased sensitivity to 80μg/ml 4-HC toxicity by 23 to 50% over that seen with the control cells with no prior ATRA incubation.

INCREASED ACETALDEHYDE CYTOTOXICITY AFTER ATRA INCUBATION

Because of the use of carotinoids derivative in cancer prevention and because acetaldehyde is a known carcinogen metabolized by ALDH, we examined the effects of the ATRA induced decrease in ALDH enzyme activity on the cytotoxicity of acetaldehyde. Figure 8A shows partial results of the dose dependent toxicity against A549 cells after 24 hr exposure to acetaldehyde. Doses of 200, 400, and 800 μM had zero toxicity. Figure 8B shows significant ($P<0.025$) increase in the cytotoxic effects of acetaldehyde (20mM for 24 hr) on A549 cells after 72 hr incubation with any of the three retinoic acids used here.

While the concentrations of acetaldehyde used here seem very high, it is important to know that A549 cells are relatively resistant and that treatment with

5-20 mM acetaldehyde for 24 hr is 100% lethal to an immortalized normal epithelial cell line IB-3 (Flotte et al., 1992) that has minimal ALDH activity (data not shown).

DISCUSSION

ALDH-1A1 has been shown to catalyze retinaldehyde into retinoic acid in several tissues (Bhat et al., 1995; Chytil, 1996; Gudas et al., 1994; Hsu et al., 1999; Lee et al., 1991; Means and Gudas, 1995; Yoshida et al., 1992). In this report we show that incubation of lung cancer cell lines with any of three different retinoic acids, results in significant decrease in ALDH-1A1 and ALDH-3A1 protein and enzyme activity. The decrease seems to take place at the post-translational level since retinoic acid did not affect the mRNA levels for both enzymes. Although significant decrease in ALDH activity is seen with very low 10^{-10} M retinoic acid concentrations, further dose escalation did not show significant drop in activity until toxic ATRA concentrations of 10^{-5} M. These observations are significant since 10^{-5} M concentration of ATRA is clinically unattainable and unfeasible. Furthermore, it seems that the decrease in ALDH activity is significant with a wide-range of ATRA concentrations. Using therapeutic concentration of ATRA (1-2 x 10^{-6} M) over 8 days show that the decrease in ALDH enzyme activity occurs as early as 12 hours after the initiation of retinoic acid exposure and continue to drop as late as 8 days, but the best and most consistent effect on 4-HC cell-sensitivity seems to be between days 3-5 of retinoic acid incubation. Such plateau effect on the 4-HC sensitivity could be related to other compensatory mechanisms or an artifact of the culture system.

The viability data (Figure 3) show minimal direct toxicity by ATRA itself, but more pronounced inhibition of proliferation (Figure 4). This observation suggests a possible cell cycle inhibition during prolonged incubation with ATRA.

These ATRA effects which may vary from one cell type to another have been described and published previously in the literature (Sun et al., 1999; Lutz et al., 2001; Sun and Lotan, 2002; Hayashi et al., 2003). It is possible that the above mentioned plateau effect on 4-HC toxicity beyond 5 days of continuous incubation with ATRA is associated to such proliferation inhibition.

We believe that this is the first report to describe a mechanism by which retinoic acid may enhance the efficacy of a chemotherapeutic agent such as 4-HC. However, few previous studies reported such effects of retinoic acid on other cytotoxic drugs including cisplatin, etoposide, paclitaxel and gemcitabine (Formelli and Cleris, 1993; Grunt et al., 1998; Kalemkerian and Ou, 1999; Pettersson et al., 2001; Shalinsky et al., 1996). The basis for those studies was the known anti-tumor effects of retinoic acids. Thus it was important to study the specificity of the ATRA-induced enhancement of the cytotoxic effects of 4-HC against A549 cells. Our studies show that ATRA pre-incubation results in increased sensitivity to melphalan and PKP, both are not metabolized by ALDH-1 or ALDH-3, however, to a much lesser degree than that seen with 4-HC. Thus, ATRA or other retinoic acids seem to have additional yet unknown effect other than that on ALDH. These results are not surprising because retinoids have been known for years as potent agents that control cellular proliferation, differentiation and apoptosis through a wide array of signal transduction pathways.

Acetaldehyde is a known carcinogenic aldehyde that could be found in cigarette or car exhaust smoke as well as a product of ethanol metabolism.

Acetaldehyde plays an important role in the pathogenesis of tissue injury that results from alcohol and cigarette consumption (Feron et al., 1991; Koivisto and Salaspuro, 1997). Since acetaldehyde is a substrate for aldehyde dehydrogenase, the presence of high levels of ALDH could be protective against the toxicity of acetaldehyde, and vice versa. Our studies show that the ATRA induced reduction in ALDH-1A1 and ALDH-3A1 results in increased acetaldehyde toxicity. Similar interaction was reported between β-carotene and acetaldehyde and was attributed to the common metabolic pathway possibly via ALDH (Ni et al., 2001).

Our current results should open the way to new studies in order to further evaluate the regulatory mechanisms involved in the retinoic acid-induced reduction in ALDH-1A1/ALDH-3A1 levels. The fact that the ALDH-1A1/ALDH-3A1 mRNA's were not reduced by ATRA indicates a possible post-translational mechanism that result in decrease protein levels and enzyme activity of these isozymes. It is possible that our starting hypothesis about a retinoic acid-mediated negative feedback might be true, but the possibility that ALDH inhibition may be induced indirectly through other retinoic acid known biological effect seems more likely and further investigation will be needed. Indeed, retinoic acid was reported to reduce p11 protein (Gladwin et al., 2000) cyclin D1 (Langenfeld et al., 1997), and retinoic acid receptors α and β by inducing degradation through the ubiquitin-proteasome pathway, a post-translational mechanism. Thus, it is possible that ALDH-1A1 and ALDH-3A1 are also regulated by proteolysis through the ubiquitin-proteasome system or other mechanisms and that the

retinoic acid effect on their levels is mediated through increased protein degradation.

In summary, we have shown that different forms of retinoic acids reduce the ALDH-1A1 and ALDH-3A1 proteins and enzyme activity via a post-translational mechanism and increase the cytotoxicity of 4-HC and acetaldehyde in lung cancer cell lines. Based on these observations, it will be reasonable to pursue clinical trials combining ATRA and cyclophosphamide in the treatment of lung cancer and other malignancies. Furthermore, our observations could serve as the basis for studying and revealing the regulatory mechanisms involved in determining the cellular expression of these enzymes.

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FOOTNOTES

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2. Please send reprints to: Dr. Jan Moreb, University of Florida College of Medicine, Department of Medicine, Division of Hematology/Oncology, 1600 SW Archer Road, Room R4-220, PO Box 100277, Gainesville, FL 32610-0277

FIGURE LEGENDS

Figure 1: ATRA dose response effect on ALDH enzyme activity in A549 cells. Each point on the curve represents the mean value for the proportion (%) of remaining ALDH activity compared to untreated cells after 72 hr incubation with ATRA. The decrease in activity was highly significant in all ATRA concentrations used ($P < 0.005$).

Figure 2: Western blot analysis shows decrease in ALDH-1A1 and ALDH-3A1 proteins at different time points of incubation of A549 cells with 10^{-6} M ATRA. ATRA was added every 48 hr for continued effect. Control (C) A549 cells were treated with equivalent volume of ethanol because it was used to dissolve the ATRA.

Figure 3: Effect of ATRA incubation on A549 Cell Viability. 10^5 cells/experimental group were incubated with (dark bars) or without (light bars) 10^{-6} M ATRA for 3, 5 and 8 days. Cell viability was determined by trypan blue exclusion criteria. Each bar represents mean ± SD of viability measured in $\geq$3 similar experiments.

Figure 4: Effect of ATRA on A549 cell proliferation as reflected by the fold increase of total viable cells over time. 10^5 cells/group were incubated with (dark bars) or without (light bars) 10^{-6} M ATRA for 3, 5 and 8 days. Cell count and viability was determined at each time point and the fold increase in viable cell number was assessed in comparison to the starting cell number. Each bar represents mean ± SD of the fold increase in cell number from at least 3 different

experiments. The differences between the ATRA and the control groups were significant on days 5 and 8 of incubation ($P < 0.025$).

Figure 5: Effect of ATRA incubation on ALDH enzyme activity in two different lung cancer cell lines.

Figure 6: Two different assays showing the effect of ATRA on 4-HC toxicity. **Panel A**. LDH Cytotoxicity Assay: Effect of 72 hr preincubation with different retinoic acids (at 10^{-6} M concentration) on 4-HC toxicity against A549 cells. After incubation with ATRA (checked bar), 13-cis retinoic acid (clear bar), or 9-cis retinoic acid (black bar), cells were treated with either 0, 40 or 80 μg/ml 4-HC as described in the Methods, and then 24hr later, LDH cytotoxicity assay was performed to determine 4-HC toxicity. The % release of LDH from the 0 μg/ml 4-HC treatment groups was calculated in relation to the LDH readings in the culture medium only.

Panel B. Liquid Colony Assay: After 72 hr incubation with (black bar) or without 10^{-6} M ATRA and treatment with 4-HC, A549 cells were washed x2 and plated at 2.5×10^4 cells/ml/plate, using RPMI culture medium plus 10% FBS. Adhered colonies were scored on day 4. The results reflect the recovery of colonies after 4-HC treatment as a percentage of the number of colonies counted from the same number of untreated cells. Untreated ATRA and Control cells had similar (mean ± SD) number of colonies obtained from plating 200 cells/ml/plate, 47 ± 10 and 47 ± 9, respectively.

Figure 7: Effect of ATRA concentrations on ALDH enzyme activity (A) and the effect on 4-HC toxicity (B) in A549 cells. $5x10^5$ cells/experimental group were incubated with ATRA for 72 hr and then cells were treated with 4-HC (80 μg/mL) as described in the Methods. LDH cytotoxicity assay was performed 24 hr later. The results reflect mean ± SD of 3 different experiments. The increase in 4-HC toxicity was significant only after incubation with 10^{-7} and 10^{-6} M of ATRA ($P < 0.01$).

Figure 8: **Panel A.** Acetaldehyde dose dependent cytotoxicity against A549 cells. $5x10^5$ cells/experimental group were treated for 24 hr with varying doses of acetaldehyde and toxicity measured by LDH cytotoxicity assay. **Panel B.** Effect of ATRA preincubation on the sensitivity of A549 cells to Acetaldehyde (20 mM/24 hr). $5x10^5$ cell/experimental group were incubated with 10^{-6} M of either ATRA, 13-cis retinoic acid (RA), 9-cis RA or just culture medium (control) for 72 hr and then 10^5 cells of each group were incubated with 20mM acetaldehyde for 24 hr. Toxicity was measured by LDH cytotoxicity assay and the results were expressed as mean ± SD of 3 similar experiments. For LDH release from cells in the 4 groups prior to acetaldehyde treatment, see Figure 6. All differences between treatment groups and control group shown in this figure were highly significant with $P < 0.025$.

Table 1: Effect of Retinoic Acid on ALDH activity in A549 Lung Cancer Cells

ATRA	Day 3 (n = 7)[a]	Day 5 (n = 3)	Day 8 (n = 2)
% of Control	41 ± 11[b] 46 ± 5	51 ± 8 57 ± 13	71 ± 7 68 ± 6
13 Cis Retinoic Acid	(n = 2)	(n =1)	(n = 1)
% of Control	35 ± 5 46 ± 4	31 ± 3 28 ± 3	54 ± 3 32 ± 4
9 Cis Retinoic Acid	(n = 2)		
% of Control	43 ± 8 30 ± 11		

[a] n = No. of experiments

[b] Values represent the mean ± SD of the % decrease in ALDH activity in cells treated with retinoic acid (10^{-6} M) in comparison to untreated A549 cancer cells. The results of days 5 and 8 for 13 Cis retinoic acid reflect the mean ± SD of 4 readings from one experiment. In each raw, the top value is calculated based on activity measured as nmole/ mg protein.min, while the bottom value is calculated based on activity measured as nmole/ 10^7 cells.min

Table 2: Effect of Preincubation of A549 Lung Cancer Cells with Retinoids on 4-HC Toxicity

	Control		ATRA		13-Cis Retinoic Acid	
4-HC dose (µg/ml)	40	80	40	80	40	80
3 Days	28 ± 2[a]	56 ± 1	71 ± 5[b]	94 ± 4[b]	71 ± 4[b]	91 ± 0[b]
5 Days	38 ± 3	73 ± 3	77 ± 8[b]	89 ± 3[b]	68 ± 3[b]	74 ± 2
8 Days	49 ± 3	75 ± 3	67 ± 0[b]	74 ± 2	55 ± 1[b]	87 ± 4[b]

[a] A549 cells were incubated with or without 10^{-6}M ATRA or 13-Cis retinoic acid for 3, 5, and 8 days and then 10^5 cells/group were treated with 4-HC (40 and 80 µg/ml) for 30 min at 37ºC. Cells were then washed x2 and plated in liquid culture. 24 hr later, LDH cytotoxicity assay was performed to determine 4-HC toxicity. The values given reflect the mean ± SD of the % toxicity from at least 2 different experiments (3 readings per each experiment) for ATRA and only one for 13-cis retinoic acid. At 0 µg/ml of 4-HC, there was no significant difference in LDH release between the 3 groups, even after 8 days of incubation

[b] $P < 0.05$ when compared to the corresponding Control.

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Table 3: Effect of ATRA Preincubation on PKP and Melphalan Toxicity Against A549 Cells in Comparison to That of 4-HC

ATRA[a] Concentration	Cytotoxicity %		
	PKP	Melphalan	4-HC
0	49 ± 8	4 ± 6	22 ± 6
$2x10^{-10}$ M	50 ± 7	4 ± 5	22 ± 7
$2x10^{-8}$ M	51 ± 6	5.5 ± 2	26 ± 4
$2x10^{-6}$ M	55 ± 22	10 ± 2 [b]	73 ± 7 [c]

[a] ATRA preincubation for 72 hours, then treatment with either PKP (60 μg/ml), melphalan (60μM) or 4-HC (80 μg/ml). Cytotoxicity (% LDH release) was measured about 24 hours after the treatment with the chemotherapeutic agents. The values shown reflect mean ± SD of ≥ 6 experiments.

[b] $P < 0.05$.

[c] $P < 0.0001$

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Figure 1

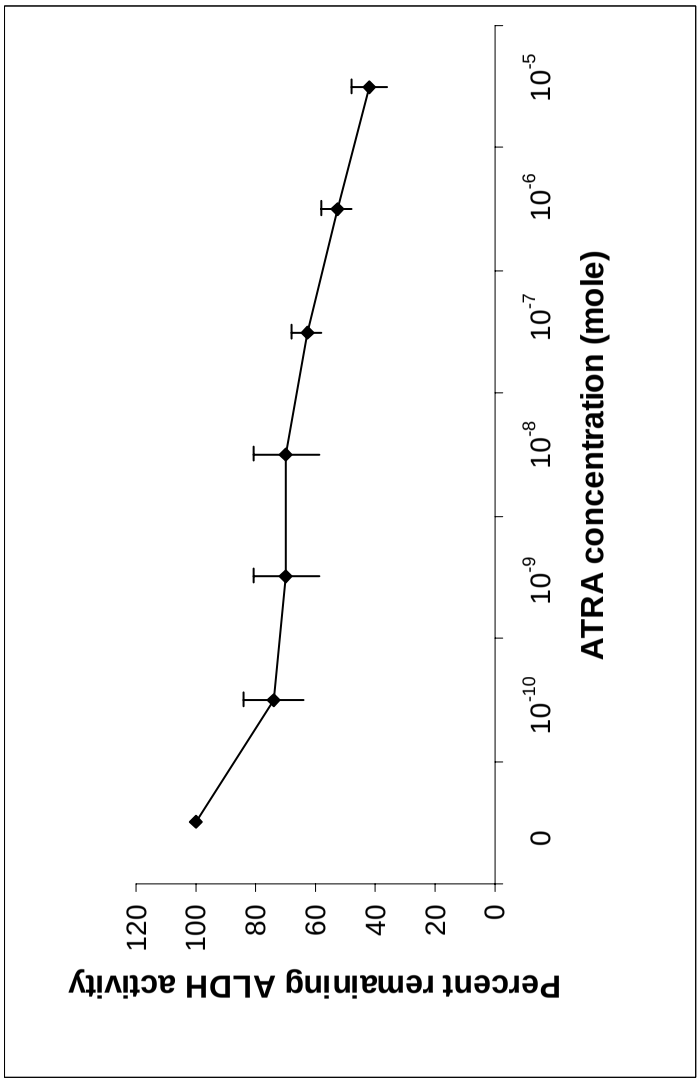

Figure 2

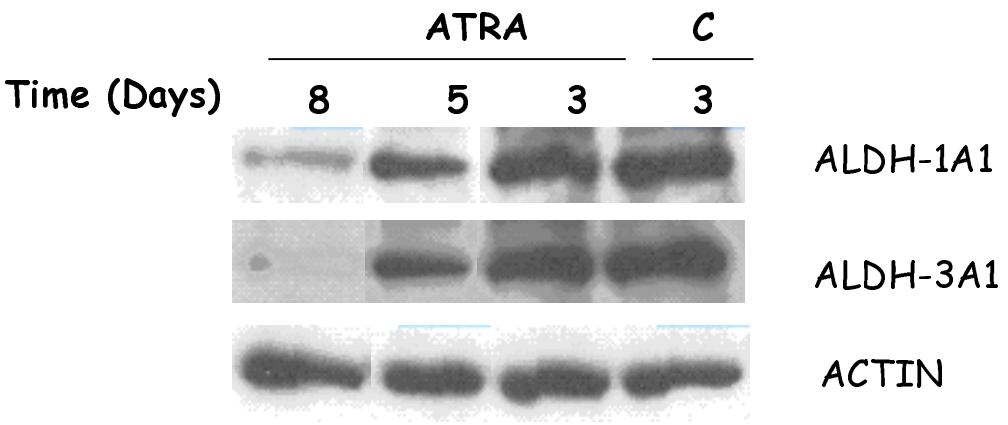

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Figure 3

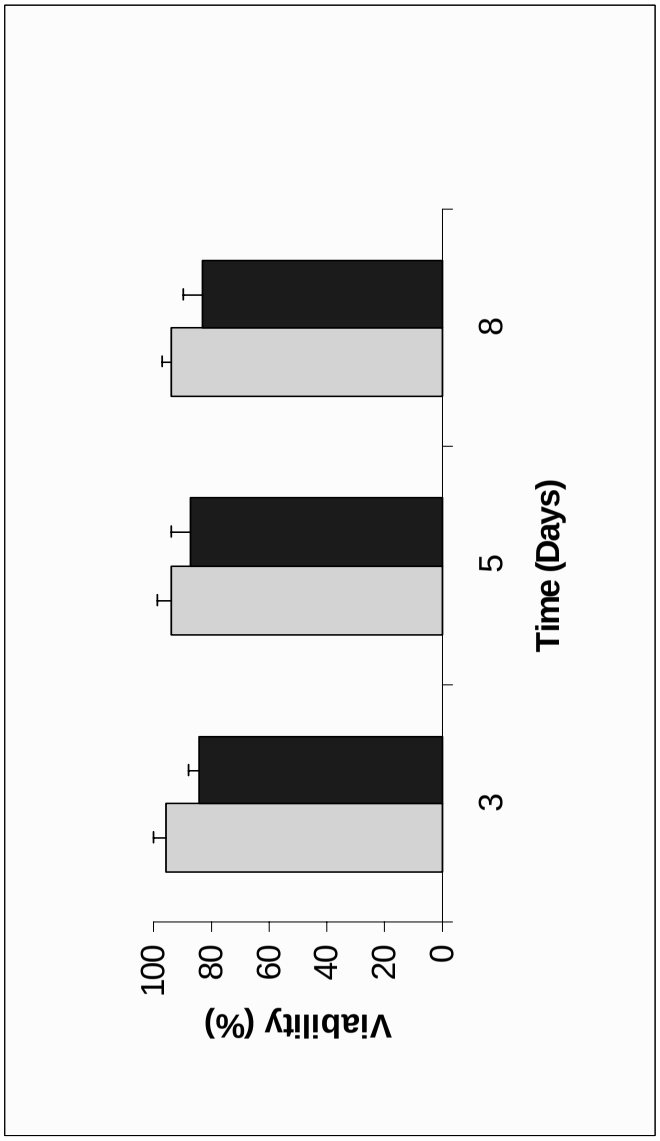

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Figure 4

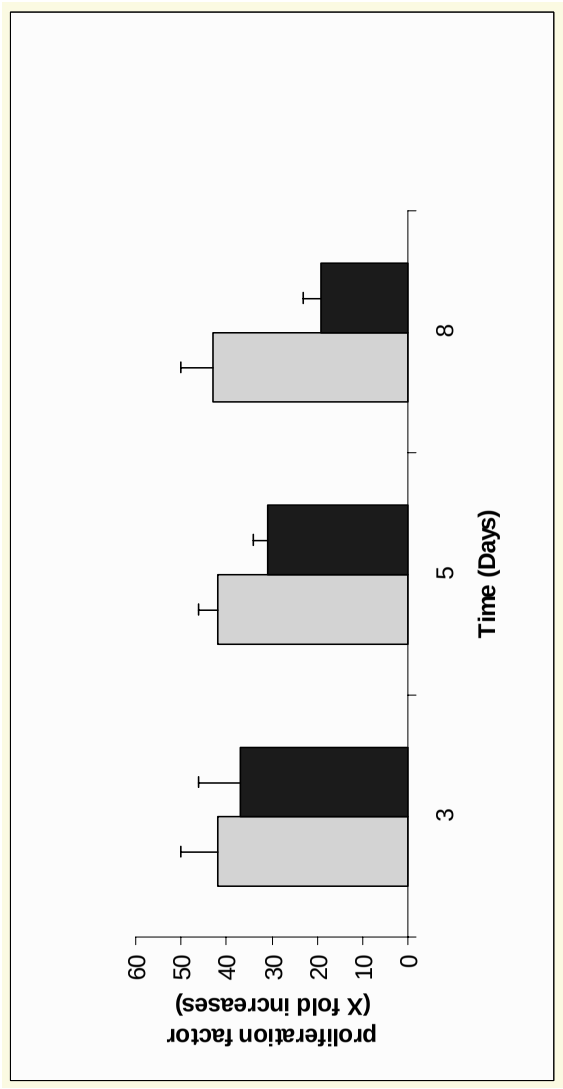

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Figure 5

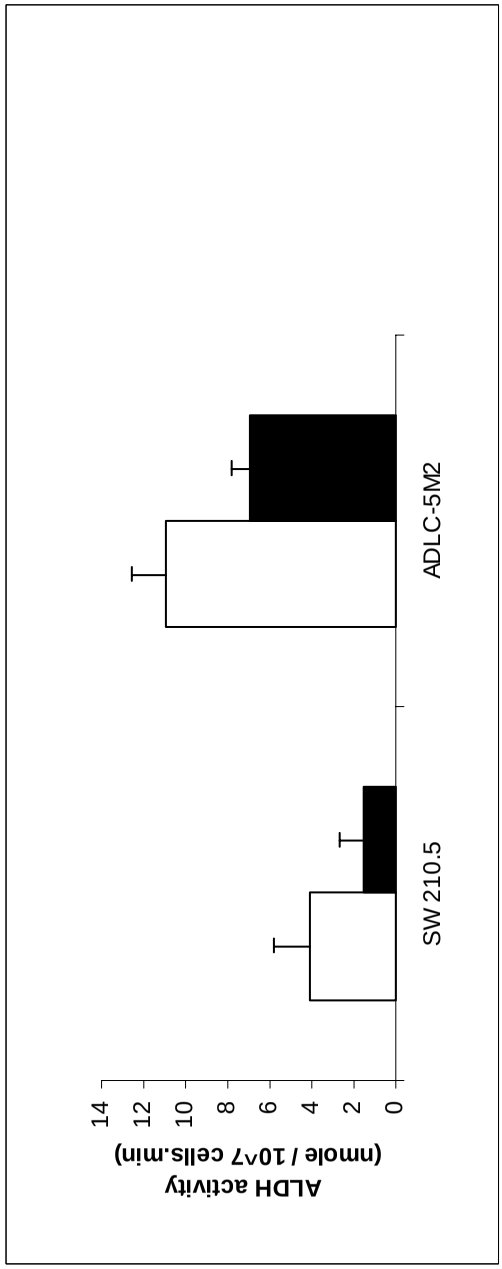

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Figure 6

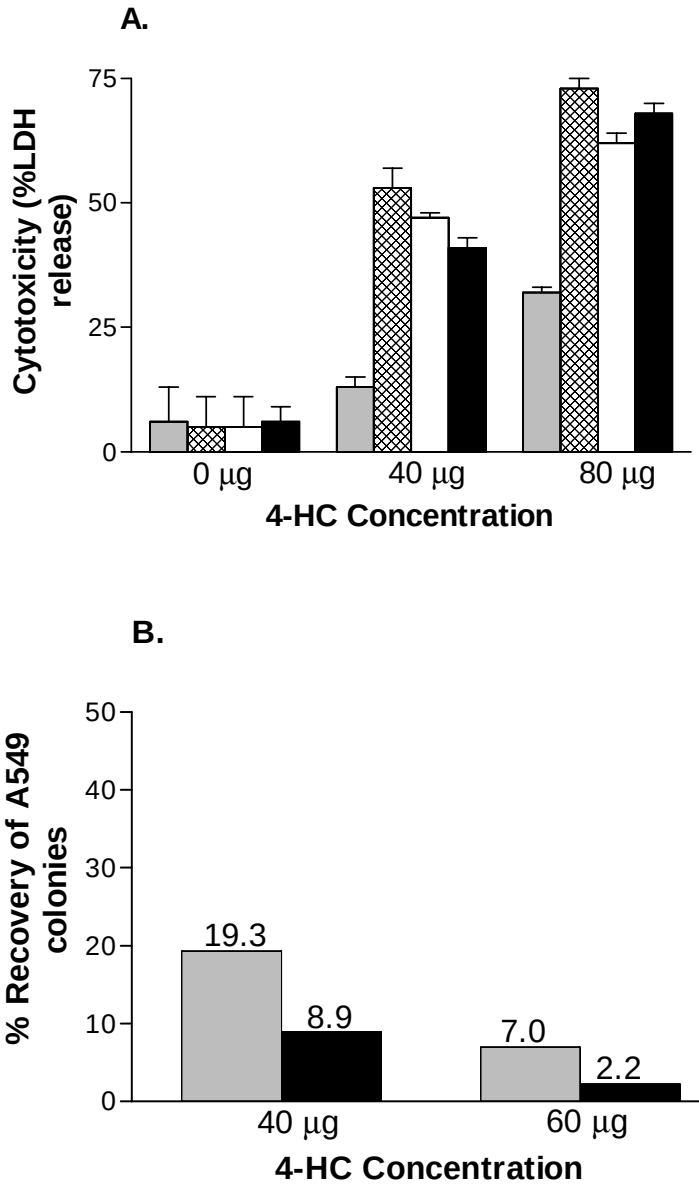

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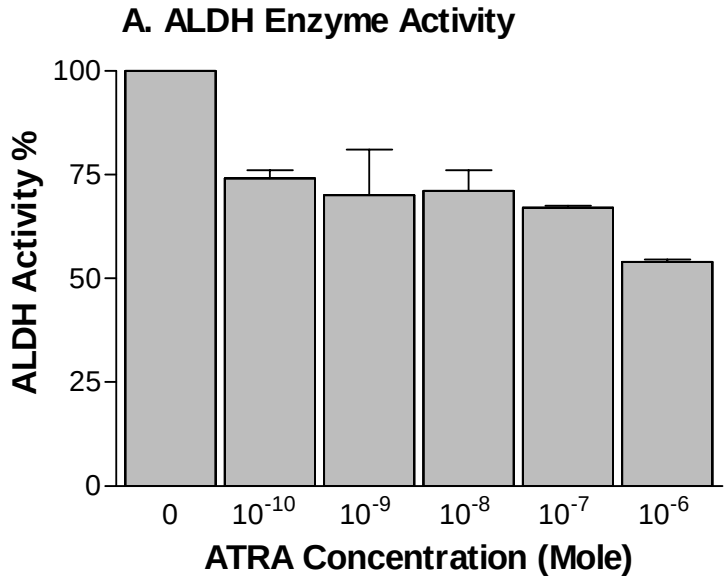
Figure 7



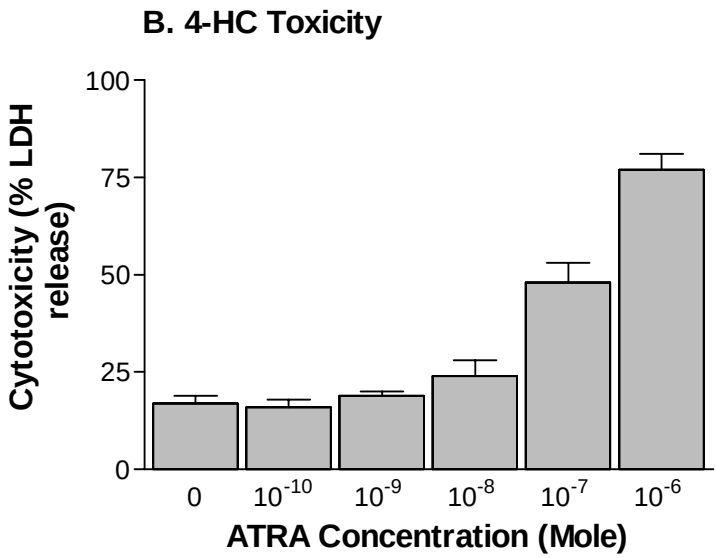


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Figure 8