Expression of Antisense RNA to Aldehyde Dehydrogenase Class-1 Sensitizes Tumor Cells to 4-Hydroperoxycyclophosphamamide In Vitro¹

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ABSTRACT

Previous studies in this laboratory showed that the overexpression of human aldehyde dehydrogenase class-1 (ALDH-1) with a retroviral vector resulted in increased resistance to 4-hydroperoxycyclophosphamide (4-HC), an active metabolite of cyclophosphamide. The present study examined the effect of ALDH-1 antisense RNA expression on ALDH-1 activity and sensitivity to 4-HC toxicity. Three different ALDH-1 cDNAs were synthesized that are either missing the N terminus (N), C terminus (C), or both (NC) and subcloned into the BamHI cloning site of pLXSN retroviral vector in the antisense (AS) orientation (AS-N, AS-C, and AS-NC, respectively). It was demonstrated that the overexpression of each of the AS constructs in K562 leukemic cells and A549 lung cancer cells results in suppression of ALDH-1 mRNA and enzymatic activity. Furthermore, the AS-N and AS-NC were generally more effective than AS-C in reducing the ALDH-1 activity. Both K562 and A549 cells expressing the ALDH-1 AS became significantly more sensitive to 4-HC toxicity as demonstrated by clonogenic and liquid culture assays. The increase in 4-HC sensitivity was in correlation with the degree of suppression of ALDH-1 activity. Moreover, such increase in 4-HC sensitivity, especially with AS-N and AS-NC, was to a similar degree seen with the use of diethylaminobenzaldehyde, a specific inhibitor of ALDH-1. These results indicate that ALDH-1 expression and activity can be specifically and effectively suppressed by AS RNA and lead to increased sensitivity to 4-HC.

Aldehyde dehydrogenase class-1 (ALDH-1) and class-3 (ALDH-3) are established molecular determinants of sensitivity to oxazaphosphorines, a group of important anticancer drugs that include cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC), mafosfamide, ifosfamide, and 4-hydroperoxyifosfamide. Both enzymes have been shown to catalyze the detoxification of these drugs (Sreerama and Sladek, 1997). The overexpression of either ALDH-1 (Magni et al., 1996; Moreb et al., 1996, 1998) or ALDH-3 (Bunting and Townsend, 1996) has been shown to induce resistance to 4-HC or mafosfamide in vitro. These studies have implications for the use of these enzymes in gene therapy strategies in vivo mainly by increasing resistance of normal hematopoietic progenitors and thus increasing the therapeutic index of the oxazaphosphorine drugs.

With the same technology of gene transfer, a different strategy can be pursued in which tumor cells are manipulated to down-regulate the expression of ALDH-1 and thus become more sensitive to treatment with oxazaphosphorines. One such strategy is to use antisense (AS) therapy to suppress ALDH-1 protein production. Current strategies of AS therapy target oncogenes or drug-resistance genes (Mercola and Cohen, 1995; Zhang, 1996), with either AS oligonucleotides or in vivo expression of AS molecule delivered by a vector. Advantages and disadvantages of each approach have been discussed in Mercola and Cohen (1995), Tonkinson and Stein (1996), Zhang (1996), and Crucio et al. (1997). One of the advantages for gene transfer approach is the theoretical ability to selectively target the AS expression in tumor cells.

Targeting the expression of ALDH-1 can be of great clinical significance due to its presence in many tumor types (Sreerama and Sladek, 1997) and due to the fact that oxazaphosphorines are widely used effective drugs. This study focuses on the effects of ALDH-1 AS RNA on ALDH-1 expression and activity and subsequently on the resistance to 4-HC with K562 leukemic cells and A549 lung cancer cells in vitro.

Received for publication November 1, 1999.

¹ This study was supported by Grant R29-CA59684 awarded by the National Cancer Institute (to J.S.M.).

ABBREVIATIONS: ALDH-1, aldehyde dehydrogenase class-1; 4-HC, 4-hydroperoxycyclophosphamide; AS, antisense; NSCLC, nonsmall cell lung cancer; FBS, fetal bovine serum; bp, base pair; WT, wild type; ECL, enhanced chemiluminescence; dCTP, deoxy cytidine 5’-triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DEAB, diethylaminobenzaldehyde; CP, cyclophosphamide; CYP, cytochrome P450.
Our results show that ALDH-1 AS successfully decreases ALDH-1 expression and increases sensitivity to 4-HC.

Materials and Methods

Cell Cultures. K562 leukemia and A549 nonsmall cell lung cancer (NSCLC) cell lines were obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in liquid culture with RPMI 1640 culture medium with 10% fetal bovine serum (FBS) and used when in the exponential log phase of their proliferation. TF-1, a leukemic cell line known for its high constitutive expression of ALDH-1, was used as a positive control.

ALDH-1 AS cDNA Synthesis and Construction of Expression Vector. Generation of the ALDH-1 cDNA with truncated 5' and 3' termini was performed by polymerase chain reaction with primers containing BamHI cloning sites and the 1570-base pair (bp) cloned ALDH-1 cDNA template (Moreb et al., 1996). The actual ALDH-1 cDNA is 1506 bp in length, including translational start and stop codons. The difference between 1570 and 1506 is the result of a 64-bp 5' extension that is part of the nontranslated leader sequence for ALDH-1. Three different AS cDNAs were synthesized as follows. The 1422-bp 3' truncated ALDH-1 cDNA was synthesized by using the 5' primer (5'-GGATCCGGATCAGAACCAAATTGCTGAC-3') and the 3' primer (5'-GGATCCACTGTTCCTGCCT-3'), which removed 148 bp 5' of the translational stop codon or the C terminus (AS-C); the 1351-bp 5' truncated ALDH-1 cDNA was synthesized with the 3' primer (5'-GGCCATCCCGTTATGAGTTCTTCTTCGAGAAG-3'), which removed 219 bp from the 5' end, including the 64-bp extension and 155 bp 3' of the translational start codon or the N terminus (AS-N); and the 1203-bp 5'/3' truncated ALDH-1 cDNA was synthesized with the 5' primer (5'-GGATCCAGAAGGAGATAGGAGAAG-3'), which removed 219 bp from the 5' end, including the 64-bp extension and 155 bp 3' of the translational start codon as well as the 3' primer (5'-GGATCCACACTGTTCCTGCCT-3'), which removed 148 bp 5' of the translational stop codon (AS-NC).

In each case, the polymerase chain reaction fragments were cloned into the Novagen pT7 Blue T-vector and mapped by restriction digestion before being subcloned into the BamHI cloning site of the pLXSN retroviral vector (Miller and Rosman, 1989) whose restriction map has been previously published (Moreb et al., 1998). The pLXSN truncated ALDH-1 cDNA clones oriented in the AS direction and the long terminal repeat promoter as determined by digestion with BglII, EcoRI, and HindIII, were expressed in the TF-1, leukemic cell line known for its high constitutive expression of ALDH-1, was used as a positive control.

ALDH-1 enzyme activity was monitored at 340 nm in a Beckman DU-70 spectrophotometer at 37°C. A total of five readings of ALDH-1 activity was obtained each time for each experimental group. A control reaction in which propionaldehyde was not added monitored the endogenous rate of NAD+ reduction.

Western Blot Analysis. Western blot analysis was performed to demonstrate changes in the protein levels of ALDH-1 and ALDH-3. However, due to low levels of expression of both enzymes in K562 cells, adequate Western analysis was possible only for A549 cells. Protein was extracted from cell lysates as described above, and equal amounts for each experimental group were size separated in a 10% denaturing SDS-polyacrylamide gel. Three identical gels were performed. One was stained with Coomassie blue to verify equal protein loading and the other two were electrophoresed onto nitrocellulose membranes that were blocked for 1 h in 5% nonfat dry milk dissolved in Tris-buffered sodium containing 0.05% Tween 20. The specific proteins were then visualized with either chicken anti-human ALDH-1 or ALDH-3 primary antibodies (supplied generously by Dr. Norman Sladek, University of Minnesota, Minneapolis) at 1:400 dilution, secondary antibody (horseradish peroxidase-labeled rabbit anti-chicken antibody; Sigma Chemical Co., St. Louis, MO) at 1:8000 dilution, and the enhanced chemiluminescence (ECL) method (ECL Western blotting kit; Amersham, Arlington Heights, IL).

Southern Analysis. DNA was extracted and quantitated from K562 cells of the four experimental groups (WT, AS-N, AS-NC, and AS-C) as described in Moreb et al. (1998). To determine genomic integration of the AS cDNAs, 25 μg of DNA from each group was digested with BamHI, BstXI, EcoRI, and HindIII, then separated on a 0.8% agarose gel. After capillary blotting onto a nylon membrane (MSI, Westborough, MA) in 20× standard saline citrate overnight, the membrane was exposed to UV (Stratalinker UV Crosslinker 1800; Stratagene, La Jolla, CA) and then baked at 80°C for 2 h. The blot was hybridized overnight at 65°C with random-primer [32P]dideoxy cytidine 5'-triphosphate (dCTP)-labeled ALDH-1 cDNA, and washed three times at room temperature before being autoradiographed. Blots were exposed to X-ray film with two intensifying screens at ~80°C.

Northern Analysis. Northern analysis was used to detect expression of the AS RNA and its effect on ALDH-1 mRNA. Total RNA was extracted from K562 cells of each experimental group with the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). Total RNA (20 μg/lane) was separated on 1.2% agarose formaldehyde gel and blotted onto a nylon membrane (MSI) by capillary transfer for 2 days. The blots were hybridized overnight at 42°C with random-primer [32P]dCTP-labeled ALDH-1 cDNA, and washed with 0.2× standard saline citrate and 0.1% SDS first at room temperature and afterward at 56°C before being autoradiographed. Blots were exposed to X-ray film with two intensifying screens at ~80°C. These same blots were stripped and reprobed twice, once with Neo cDNA for correlation of Neo and ALDH-1 expression, and another with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, to assess equal loading of RNA or any nonspecific effects related to the AS expression.

In Vivo 4-HC Treatment Assay. Diethylaminobenzaldehyde (DEAB), 4-HC, and phenylketophosphamide, a synthetic 4-HC analog not metabolized by ALDH-1, were generously supplied by Dr. Michael Colvin (Duke University, Durham, NC) and kept at ~20°C until used. A solution of 1 μg/μl 4-HC was prepared in culture medium, filter sterilized (0.2 μm), and kept on ice immediately before use. DEAB (an ALDH-1 inhibitor) also was prepared 10 to 15 min before use. To investigate the effect of AS RNA on sensitivity to 4-HC, K562 cells (5 × 105 cells/ml) or A549 cells (2.5 × 104 cells/ml) from each experimental group (WT, AS-N, AS-NC, and AS-C) were treated with 4-HC and incubated for 30 min at 37°C. In some experiments, a separate WT cell group was treated with 25 μM DEAB for 15 min before the addition of 4-HC to compare the effect of the inhibitor versus ALDH-1 AS on sensitivity to 4-HC. After 4-HC treatment, cells were washed twice with chilled culture medium.
(RPMI with 10% FBS), then plated. K562 cells were plated in methylcellulose containing 25% FBS or in liquid culture in RPMI containing 10% FBS, as described in Moreb et al. (1998). Colonies were counted on day 7 of methylcellulose cultures with an inverted microscope. The total number of viable cells in liquid cultures was determined twice within a 7-day period. Viability was determined by the trypan blue exclusion criteria. A549 cells were plated in liquid colony assay with four 35-mm petri dishes per group. Colonies (>10 cells) adhered to the bottom of the plate were counted on day 4 with an inverted microscope. Untreated A549 cells were plated similarly at 250 cells/ml/dish.

**Statistical Analysis.** Statistical significance of the difference between experimental groups was calculated with Student’s *t* test for two means. A *P* value of <.05 was considered statistically significant.

### Results

**ALDH-1 Activity Measurements in K562 Cells.** To determine the effect of AS RNA on ALDH-1 expression, ALDH-1 enzyme activity was measured in all experimental groups. Because the results with WT cells and those transfected with pLXSN vector only were similar in multiple experiments for both K562 and A549 (Table 1), only the results with WT cells are reported throughout the manuscript. Furthermore, transfected cells had similar morphology and proliferation characteristics as WT cells. ALDH-1 activity was measured over a 3-month period for each AS K562 cell type. Initially, all of the AS cells showed a significant decrease in ALDH-1 activity, however, ALDH-1 activity was restored by day 40 of culture and while the cells were maintained with G418 (Table 2). Figure 1 represents the mean ± S.E. of the activity measured over several weeks of culture for each experimental group. The ALDH-1 activity in AS-N and AS-NC cells was significantly lower than that for WT cells (*P* < .001). Although reduction in ALDH-1 activity was seen in the AS-C cells as well (Table 2), it did not reach significance when the overall mean was compared with that of the WT cells. This is most likely explained by lack of consistent effect of AS-C on blocking the translation of ALDH-1 protein and fluctuation in its activity over time in the AS-C K562 cells.

**ALDH-1 AS Integration and Expression in K562 Cells.** In view of the reduction in ALDH-1 activity, and to verify that this reduction is indeed the result of the ALDH-1 AS cDNA genomic integration and mRNA expression, several studies were performed. Figure 2 shows a Southern analysis in which DNA from the different K562 experimental groups was digested with four different restriction enzymes, blotted, and labeled with 32P-labeled ALDH-1 cDNA. Digestion with BamHI, the cloning site for ALDH-1 cDNAs in the pLXSN vector, reveals two DNA bands that were common among all groups and most likely represent the genomic ALDH-1 DNA. Other bands of different sizes (1351, 1422, and 1203 bp) represent the AS ALDH-1 cDNAs: AS-N, AS-C, and AS-NC, respectively. Digestion with EcoRI, which cuts once in the vector upstream to the ALDH-1 cDNA, and HindIII, which cuts once in the vector downstream to the ALDH-1 cDNA, both showed two extra bands with all AS groups compared with WT DNA, indicating two different integration sites. Digestion with a noncut restriction enzyme, BsrXI, resulted in at least two DNA fragment larger than the vector that are not seen in the WT DNA and are different in size among the different AS clones. Overall, these results demonstrate the genomic integration of the AS vector constructs in two different sites.

Northern analysis was performed to detect the presence of retrovirally transcribed ALDH-1 AS mRNA in the different K562 experimental groups (Fig. 3). The results indicate the presence of the expected AS mRNA in all three AS K562 cells (Fig. 3, top, top band), whereas the WT ALDH-1 mRNA [top, lower band (∼3 kb)] can be seen in the TF-1 cells (first lane) and WT K562 (second lane) but not detected in the cells expressing the ALDH-1 AS mRNA. Total RNA from TF-1 leukemic cells was included as a positive control for WT ALDH-1 because these cells are known to have high levels of WT ALDH-1 mRNA (Moreb et al., 1995, 1998). Neomycin resistance gene was detected only in the AS transfected K562 cells (Fig. 3, middle).

Figure 4 shows the results of similar Northern analysis as in Fig. 3, however, the RNA was obtained from the same K562 experimental groups at a later time of cell culture that corresponded to the increase in the ALDH-1 activity in the three AS experimental groups while cultured in the absence of G418 (Table 2). Figure 4 shows the loss of AS mRNA in all cell lines (AS-C, AS-N, and AS-NC) with rebound increase in the WT ALDH-1 mRNA. These findings correlate very well with the increase in ALDH-1 activity shown in all these cells in Table 2, and confirm the loss of the retrovirally transfected ALDH-1 AS expression. The neomycin resistance gene (Fig. 4, middle) also was undetectable. However, GAPDH expression was not affected by the expression of ALDH-1 AS or lack of it (Figs. 3 and 4).

**ALDH-1 AS Overexpression Sensitizes K562 Cells to 4-HC.** To determine whether ALDH-1 AS RNA expression led to increased sensitivity to 4-HC, K562 cells, either WT or in which DNA from the different K562 experimental groups was digested with four different restriction enzymes, blotted, and labeled with 32P-labeled ALDH-1 cDNA. Digestion with BamHI, the cloning site for ALDH-1 cDNAs in the pLXSN vector, reveals two DNA bands that were common among all groups and most likely represent the genomic ALDH-1 DNA. Other bands of different sizes (1351, 1422, and 1203 bp) represent the AS ALDH-1 cDNAs: AS-N, AS-C, and AS-NC, respectively. Digestion with EcoRI, which cuts once in the vector upstream to the ALDH-1 cDNA, and HindIII, which cuts once in the vector downstream to the ALDH-1 cDNA, both showed two extra bands with all AS groups compared with WT DNA, indicating two different integration sites. Digestion with a noncut restriction enzyme, BsrXI, resulted in at least two DNA fragment larger than the vector that are not seen in the WT DNA and are different in size among the different AS clones. Overall, these results demonstrate the genomic integration of the AS vector constructs in two different sites.

### TABLE 1

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>K562</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>pLXSN</td>
</tr>
<tr>
<td>+G418</td>
<td>2.64 ± 0.3</td>
<td>1.09 ± 0.45</td>
</tr>
<tr>
<td>−G418</td>
<td>2.47 ± 0.4</td>
<td>2.44 ± 0.50</td>
</tr>
</tbody>
</table>

* Enzyme activity is expressed in nanomoles per 10^7 cells per minute. Data represent mean ± S.D. of 15 measurements performed at weekly intervals for three consecutive weeks. Measurements were performed on the same cells growing in liquid culture with 1 mg/ml G418 (+G418) and after ~6 weeks of culture in the absence of G418 (−G418).

b Significantly different compared with WT group, *P* < .05.

### TABLE 2

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>WT</th>
<th>AS-N</th>
<th>AS-C</th>
<th>AS-NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>+G418</td>
<td>2.69 ± 0.3</td>
<td>1.09 ± 0.45</td>
<td>1.42 ± 0.8</td>
<td>1.25 ± 0.2</td>
</tr>
<tr>
<td>−G418</td>
<td>2.47 ± 0.4</td>
<td>2.44 ± 0.50</td>
<td>2.98 ± 0.6</td>
<td>2.38 ± 0.2</td>
</tr>
</tbody>
</table>
expressing ALDH-1 AS (AS-C, AS-N and AS-NC), were treated with various doses of 4-HC (10 and 15 \( \mu \)g/ml) and cultured in methylcellulose colony assay or liquid culture. Table 2 shows the results obtained from one of three representative experiments with similar results. Data represent the mean ± S.D. of the total colonies counted as well as the percentage recovery after 4-HC treatment compared with untreated cells. It is shown that cells expressing ALDH-1 AS were more sensitive to 4-HC than WT cells with AS-N and AS-NC exhibiting significantly increased sensitivity (\( P < .005 \)) compared with AS-C. These results correspond well to the ALDH-1 activity results shown in Fig. 1. Table 3 also shows that the addition of 25 \( \mu \)M DEAB 10 min before the treatment of WT cells with 10 \( \mu \)g/ml 4-HC affected 4-HC sensitivity to a similar proportion seen with AS-N and AS-NC. Furthermore, similar treatment with 15 \( \mu \)g/ml phenylketophosphamid showed no significant difference between the WT and AS cells, indicating the specificity of the ALDH-1 AS effects.
cells. Similar decrease in activity was demonstrated when other substrates, acetaldehyde and benzaldehyde, were used in the reaction (data not shown). In addition, Table 6 shows proportional increase in sensitivity to 4-HC in all AS cells. The effect of the different AS constructs expression on ALDH-1 and ALDH-3 proteins was studied with Western blot analysis. The same protein samples used for the enzyme activity were used for the Western analysis shown in Fig. 5. The results confirm those obtained by enzyme activity assay and demonstrate significant decrease in ALDH-1 protein levels in all AS groups, with some variability. However, ALDH-3 protein levels were relatively unaffected.

Discussion

The relationship between ALDH-1 and resistance to the oxazaphosphorines has been established for many years mainly through correlative studies, use of ALDH-1 inhibitors, and most recently by gene transfer studies (Kohn and Sladek, 1985; Sladek and Landkamer, 1985; Russo and Hilton, 1988; Bunting and Townsend, 1996; Magni et al., 1996; Moreb et al., 1996, 1998; Sreerama and Sladek, 1997). Because of several other potential mechanisms of resistance against oxazaphosphorines (McGowan and Fox, 1986; Yuan et al., 1990; Friedman et al., 1992; O’Connor et al., 1992), the exact contribution of ALDH-1 to that resistance in any cell type is still being debated and studied. In this report, we show that suppression of ALDH-1 expression (Figs. 3 and 5) and activity (Tables 2 and 6) with gene transfer to express ALDH-1 AS RNA in two different cell lines, results in a significant increase in the sensitivity to 4-HC, an active metabolite of cyclophosphamide. The increase in sensitivity to 4-HC in some of the cells with ALDH-1 AS was similar to that seen with DEAB, a specific inhibitor of ALDH-1.

AS strategies for gene silencing have attracted much attention in recent years and despite potential obstacles, AS technology (oligonucleotides or AS genes) has been widely used in the laboratory for studying gene function and in recent years introduced into the treatment of cancer patients. Because cyclophosphamide (CP) is one of the most commonly used alkylating agents and because one of the resistance mechanisms to CP is high levels of ALDH-1, a strategy to reduce ALDH-1 protein with gene transfer to express AS RNA in tumor cells may increase the therapeutic index of CP and lead to a higher rate of complete responses. Our studies demonstrate that such a strategy can be effective, both in K562 cells with low constitutive expression of ALDH-1 and in A549 cells with very high constitutive expression of ALDH-1, and in the presence of high levels of ALDH-3 as well. Given the promising results with A549 NSCLC cell line, an AS approach can potentially be used in the treatment of locally advanced NSCLC cells. Similar to phase I gene therapy studies already reported (Roth et al., 1996; Tursz et al., 1996), an adenoviral vector containing the ALDH-1 AS can be directly injected into locally advanced NSCLC cells, then treated with i.v. CP. Such strategy to reduce the levels of ALDH-1 may increase the therapeutic index of CP and lead to higher rates of response.

Another approach to increasing sensitivity of tumors to CP has been reported by Chen et al. (1996) and is based on the use of cytochrome P450 (CYP) gene CYP2B1, which activates the prodrug CP into its 4-hydroxy-CP active metabolite. Such
In each group, a total of 2 × 10^4 4-HC-treated cells in relation to the number of colonies obtained if similar number of untreated cells was cultured.

Effect of ALDH-1 AS expression on 4-HC sensitivity of K562 cells in liquid culture

<table>
<thead>
<tr>
<th>Days after 4-HC Treatment</th>
<th>WT</th>
<th>AS-N</th>
<th>AS-C</th>
<th>AS-NC</th>
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<tbody>
<tr>
<td>4</td>
<td>7 ± 2^a</td>
<td>19.5 ± 0.3^b</td>
<td>2.5 ± 0.8^a</td>
<td>2.4 ± 1.2^a</td>
</tr>
<tr>
<td>7</td>
<td>41 ± 19</td>
<td>1 ± 0.2^b</td>
<td>2.9 ± 1.6^a</td>
<td>3.6 ± 0.4^a</td>
</tr>
</tbody>
</table>

* Data represents mean ± S.D. of viable cell concentration (× 10^5 cells/ml) for each experimental group; WT K562 cells, K562 cells transfected with ALDH-1 AS-C, AS-N, and AS-NC. In each group, a total of 2 × 10^5 (5 × 10^4 cells/ml) cells was treated with 10 μg/ml 4-HC.

**Significantly different compared with WT group, P < .01.

**Significantly different compared with WT group, P < .005.

ND, not determined.

Effect of ALDH-1 AS RNA on the sensitivity of K562 cells to 4-HC and phenylketonuria (PKP)

<table>
<thead>
<tr>
<th>4-HC Dose</th>
<th>WT</th>
<th>AS-N</th>
<th>AS-C</th>
<th>AS-NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μg/ml</td>
<td>126 ± 19^a</td>
<td>28 ± 6^b</td>
<td>55 ± 5^a</td>
<td>74 ± 8^b</td>
</tr>
<tr>
<td>15 μg/ml</td>
<td>23 ± 2</td>
<td>ND</td>
<td>2 ± 1^a</td>
<td>9 ± 1^b</td>
</tr>
</tbody>
</table>

* Results represent mean ± S.D. of total colonies scored in four replicate methylcellulose culture plates, each plated at 5 × 10^4 4-HC- or PKP-treated cells per plate. Values in parentheses represent percentage recovery of colonies from 4-HC or PKP-treated cells in relation to the number of colonies obtained if similar number of untreated cells was cultured.

**Significantly different compared with WT group, P < .005.

**No significant differences were found between the AS groups and WT.

Effect of loss of activity of ALDH-1 AS on the sensitivity to 4-HC

<table>
<thead>
<tr>
<th>Time</th>
<th>WT</th>
<th>AS-N</th>
<th>AS-C</th>
<th>AS-NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 ± 15^a</td>
<td>143 ± 21^b</td>
<td>137 ± 13^b</td>
<td>126 ± 19^b</td>
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</tr>
</tbody>
</table>

* Results represent mean ± S.D. of total colonies scored in three replicate methylcellulose culture plates, each plated at 5 × 10^4 cells/plate after treatment with 10 μg of 4-HC. Values in parentheses represent percentage recovery of colonies from 4-HC-treated cells in relation to the number of colonies obtained if similar number of untreated cells was cultured.

**Significantly different compared with WT group, P < .025.

Effect of loss of activity of ALDH-1 AS on the sensitivity to 4-HC

is that the sense-AS duplex is protected against the ribosome’s unwinding activity during RNA translation when the AS molecule is missing the N terminus (Shaki-Eshleman and Liebhaber, 1988). Thus, in spite of good levels of AS-C mRNA (Fig. 3), AS-C was less effective in reducing the ALDH-1 activity and increasing 4-HC sensitivity in K562 cells (Fig. 1 and Table 3). Similar differences, although to a lesser degree, were seen in A549 cells (Fig. 5 and Table 6). Several other factors that determine the effectiveness of an AS molecule have been reported and include the following: 1) rate of transcription of the AS versus sense gene; 2) stability of the AS gene; 3) target site of the AS RNA; 4) rate of duplex formations; and 5) location of the AS gene versus that of the targeted gene (Mercola and Cohen, 1995; Zhang, 1996; Arndt and Hank, 1997). Any one or combination of these factors could have contributed to the different effectiveness of AS-C.

Finally, our results suggest that growing the ALDH-1 AS cells in the absence of G418 selective pressure, results in the loss of AS mRNA expression and rebound increase in the WT ALDH-1 mRNA (Fig. 4). We are not aware of any previous reports on such rebound phenomenon with the AS strategy. Although it is reversible after re-exposure to G418 in vitro, it is clearly of concern because according to our results (Table 5), it can result in the opposite effect, i.e., an increase in resistance to 4-HC or CP of the affected tumor cells. However, sensitizing the cells to the toxicity of 4-HC or CP may result in the elimination of all cells expressing the AS and, therefore, such rebound phenomenon may not be clinically significant. In vivo studies with an animal model are essential to address the significance of such phenomenon. Last, the mechanism for such loss of AS expression was most likely due to the absence of selection pressure because re-exposure to G418 resulted in the restoration of AS effect and continuous exposure to 0.4 to 1 mg/ml G418 maintains the expression of the different AS mRNAs. Furthermore, repeat Southern analysis, with DNA obtained from all the experimental groups during the loss of AS expression and digested with BamHI, continued to show the presence of the different AS cDNAs (data not shown).

In summary, we have defined ALDH-1 AS constructs that can effectively reduce the ALDH-1 expression and activity and increase sensitivity to 4-HC in vitro. Our studies again demonstrate the significant contribution of ALDH-1 to the resistance against oxazaphosphorines and, therefore, the potential clinical application of ALDH-1 AS RNA in cancer...
TABLE 6
Effect of ALDH-1 AS expression on enzyme activity and sensitivity to 4-HC of A549 cells

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>AS-N</th>
<th>AS-C</th>
<th>AS-NC</th>
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<tr>
<td>Enzyme activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmol/10^6 cells/min 4-HC treatment</td>
<td>760 ± 42^a</td>
<td>408 ± 7^b</td>
<td>464 ± 28^c</td>
<td>202 ± 12^d</td>
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<tr>
<td>40 µg/ml</td>
<td>126 ± 8 (1.5)^b</td>
<td>15 ± 5 (0.4)^f</td>
<td>7 ± 2 (0.16)^f</td>
<td>2 ± 2 (0.03)^f</td>
</tr>
<tr>
<td>60 µg/ml</td>
<td>18 ± 4 (0.21)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results represent mean ± S.D. of 10 enzyme activity measurements done at two different time points of cell culture.

*Results represent mean ± S.D. of total colonies scored in four replicate culture plates, each plated at 2.5 × 10^6 treated cells/plate. Values in parentheses represent percentage recovery of colonies from 4-HC-treated cells in relation to the number of colonies obtained from untreated cells.

**Significantly different compared with WT group, P < .005.


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