INHIBITION OF HUMAN ALDEHYDE DEHYDROGENASE 1 BY THE 4-HYDROXYCYCLOPHOSPHAMIDE DEGRADATION PRODUCT ACROLEIN

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ABSTRACT:

In a previous study, we observed that the elimination clearance of 4-hydroxycyclophosphamide (HCY) in patients receiving cyclophosphamide (CY) 60 mg/kg/day by 1-h i.v. infusion for 2 consecutive days decreased from day 1 to day 2 due to an apparent decrease in human aldehyde dehydrogenase 1 (ALDH1) activity. Here, the mechanism for the decrease in ALDH1 activity after CY administration was investigated. In human liver cytosol incubations, HCY inhibited ALDH activity mainly through its degradation product acrolein, whereas carboxyethylphosphoramide mustard inhibited ALDH activity only at supraclinical concentrations. Other CY metabolites evaluated, phosphoramide mustard and chloroacetalddehyde, did not inhibit ALDH. The inhibition of ALDH1 activity by acrolein in incubations with human erythrocyte ALDH1 was competitive with a $K_i$ of 0.646 $\mu$M. The inhibition was independent of preincubation time and reversible by dialysis. The percentage of inhibition of ALDH1 activity in vivo by acrolein in patients receiving CY was calculated based on the in vitro $K_i$ of acrolein, the in vitro $K_m$ of HCY, and the in vivo peak blood concentrations of HCY and acrolein. The calculations indicated that the activity of ALDH1 was inhibited by 85, 88, and 91% on days 1, 2, and 3 (24 h after the dose on day 2) of CY administration, respectively. The increase in ALDH1 inhibition with time is consistent with the decrease in HCY elimination clearance and the increase in HCY area under the plasma concentration time curve with time.

Cyclophosphamide (CY) is one of the most frequently used alkylating agents in the treatment of malignancy and in preparative regimens for bone marrow transplantation. It is a prodrug. At therapeutic concentrations of CY, it is metabolized by CYP2C9 and CYP3A4 to form 4-hydroxycyclophosphamide (HCY) or by unidentified cytochrome P-450 isoform(s) to form deschloroethylcyclophosphamide and chloroacetalddehyde (Ren et al., 1997). Deschloroethylcyclophosphamide has no antitumor effect. Chloroacetalddehyde is a potent pulmonary toxin but is formed in relatively low abundance from CY. HCY is the major active circulating metabolite. HCY enters cells and decomposes (through its tautomer aldophosphamide) to phosphoramide mustard (PM) and acrolein. PM is a bifunctional alkylator of DNA, the ultimate cytotoxic metabolite of cyclophosphamide. Alternatively, HCY is detoxified to 4-ketocyclophosphamide by cytochrome P-450 and carboxyethylphosphoramide mustard (CEPM) by aldehyde dehydrogenase 1 (ALDH1) (Dockham et al., 1992). The formation of CEPM from HCY appears to be the most important detoxifying pathway of HCY (Sladek, 1994).

In bone marrow transplantation, CY (60 mg/kg) is usually administered once a day by i.v. infusion for 2 consecutive days primarily to facilitate engraftment of donor cells, although an antitumor effect may also be obtained. In a pharmacokinetic study in bone marrow transplantation patients, we found that the area under the plasma concentration time curve of HCY increased 54.7% ($P < .002$) from day 1 to day 2 due to an increased formation clearance of HCY from CY and a decreased elimination clearance of HCY. The decreased elimination clearance of HCY apparently was caused by decreased ALDH1 activity from day 1 to day 2 as measured ex vivo in patient erythrocytes (Ren et al., 1998). ALDH1 accounts for 95% of the human liver ALDH activity forming CEPM from HCY (Dockham et al., 1992) and is the only ALDH isoenzyme present in human erythrocytes (Mathewson and Record, 1986).

The objective of this investigation was to elucidate the mechanism of the decrease in ALDH1 activity after CY administration.

Materials and Methods

Materials. Indole-3-acetaldehyde (IAL), indole-3-acetic acid (IAA), and NAD were purchased from Sigma Chemical Company (St. Louis, MO). Acrolein was purchased from Aldrich Chemical (Milwaukee, WI). 3-Aminophenol and hydroxyamine hydrochloride were purchased from Fluka Chemical (Ronkonkoma, NY). CEPM and PM were generous gifts from ASTA Medica AG (Frankfurt, Germany). 4-Hydroperoxycyclophosphamide was prepared in our laboratory by published methods (Takamizawa et al., 1975). 4-Hydroperoxycyclophosphamide (5 mM) was reduced to HCY by adding sodium thiosulfate (25 mM) and allowing the mixture to stand on ice for 1 h immediately before incubation. Isoelectric focusing gel and buffers (pH 3–10) were purchased from Novex (San Diego, CA).

Incubations. Human liver cytosol was used to screen potential inhibitors of ALDH. Human livers were obtained from the human liver bank in the Departments of Pharmaceutics and Medicinal Chemistry at the University of Washington (Seattle). Livers were homogenized in 100 mM potassium phosphate buffer (pH 7.4) and centrifuged at 10,000g, 4°C for 30 min. The
The inhibition of ALDH activity was studied in human liver cytosol incubations with IAL as the probe substrate. HCY (5 and 10 μM) caused a preincubation time- and concentration-dependent decrease of IAA formation rate (Fig. 1). The addition of NAD to the preincubation solution [20 mg/ml 3-aminophenol, 20 mg/ml hydroxylamine hydrochloride, and 10% (w/v) ferrous sulfate in 2.5 M sulfuric acid and 16.8% perchloric acid], sealed, vortexed 3 to 6 times, and centrifuged at 10,000g for 1 min at the patient’s bedside. The supernatant was transferred to a clean glass injection vial, sealed, stored in a −20°C cooler for transportation to the lab, and frozen at −70°C until analysis. On the day of analysis, the supernatant was thawed at room temperature and heated at 100°C for 2.5 min. After the sample was cooled to room temperature, 20 μl was injected into the high-performance liquid chromatography system described above. Because this assay measures acrolein before the blood is drawn and acrolein formed from HCY after the blood was drawn, HCY concentration was measured (Slattery et al., 1996) in a split sample, and the in vivo acrolein concentration was calculated as the difference between total acrolein and HCY concentration. The mean concentrations of HCY and acrolein in these five patients were used in estimating the extent to which acrolein inhibits ALDH1 activity in patients.

Data Analysis. The inhibition of IAA formation by acrolein in ALDH1 prepared from human erythrocytes was first analyzed by a Lineweaver-Burk plot. A competitive inhibition model (Segel, 1975) was then used to fit these data:

\[
\frac{V}{v} = \frac{V_{max}S}{K_m (1 + \frac{I}{K_i}) + S}
\]

The value of \(K_i\) was estimated by fitting this model to the untransformed IAA formation rate and IAL concentration data with the WinNonlin program (Scientific Consulting, Inc., Cary, NC). A weight of \(V^2\) was used in the iterative fitting process.

The percentage of inhibition of ALDH1 activity in vivo in patients by acrolein after CY administration was calculated by the following equation:

\[
100\% \times \left[1 - \frac{K_{in} + S}{K_{in} (1 + \frac{I}{K_i}) + S}\right]
\]

where the in vitro Michaelis constant of HCY (\(K_{in}\)) is 12.9 μM (Ren et al., 1998), the in vitro dissociation constant of acrolein (\(K_i\)) is 0.646 μM, the maximal blood concentration of HCY in patients (S) is 8.31, 13.9, and 0.72 μM on days 1, 2, and 3, and the maximal blood concentration of acrolein in patients (I) is 6.2, 10.2, and 7.2 μM on days 1, 2, and 3, respectively.

Results

The inhibition of ALDH activity was studied in human liver cytosol incubations with IAL as the probe substrate. HCY (5 and 10 μM) caused a preincubation time- and concentration-dependent decrease of IAA formation rate (Table 1).

Among the metabolites of CY tested (at the maximal observed clinical plasma concentration and 10 times that concentration) for inhibition of ALDH activity, only acrolein showed a pronounced effect, whereas CEPM had a modest effect and no significant inhibition was observed with PM or chloroacetaldehyde (Table 1). Thus, the mechanism of ALDH inhibition by acrolein was investigated.

The effect of acrolein on the formation of CEPM from HCY was first investigated in human liver cytosol incubations. The percentage
of inhibition of CEPM formation was $35.4 \pm 13.8\%$ and $85.3 \pm 4.1\%$ ($n = 3$), respectively, when 10 or 50 $\mu$M acrolein was added exogenously to the incubations compared with no acrolein added exogenously (Table 2). The actual mean acrolein concentrations at the beginning and the end of the incubations were 5, 15, and 55 $\mu$M in the control incubation and in the incubations with the addition of 10 and 50 $\mu$M acrolein exogenously, respectively.

Because ALDH1 is the major ALDH isoform responsible for the formation of CEPM from HCY, the mechanism of ALDH1 inhibition by acrolein was sought in incubations with ALDH1 prepared from human erythrocytes. Only one band at isoelectric point (pI) 5.2 was detected by isoelectric focusing and staining for ALDH activity in the ALDH1 prepared from human erythrocytes (Fig. 2). This pI is identical with the pI of human liver cytosolic ALDH1. Human liver homogenate and cytosol showed multiple bands, corresponding to various ALDH isoforms. The Lineweaver-Burk plot of IAA formation rate versus IAL concentration at various concentrations of acrolein and the replot of the slope indicated competitive inhibition (Fig. 3). A competitive inhibition model as described in Materials and Methods was fit to the untransformed IAA formation and IAL concentration data. The model-predicted versus the observed IAA formation rate as a function of IAL concentration at various concentrations of acrolein is shown in Fig. 4. The estimated value of $K_i$ was 0.646 $\mu$M. Preincubation of acrolein with the ALDH1 preparation for 10 min did not increase the degree of inhibition compared with no preincubation. The reversibility of the inhibition was examined by dialysis. The IAA formation rate (at 1.5 $\mu$M IAL) in the presence of 6 $\mu$M acrolein was

Human liver cytosol, HCY (20 $\mu$M), and acrolein (10, 50 $\mu$M) were preincubated at 37°C for 2 min and then the reaction was initiated with the addition of NAD (0.5 mM) and stopped after 5 min. Control incubations contained no exogenously added acrolein. Because acrolein is formed chemically from HCY during the incubations, the actual acrolein concentrations were measured at the beginning and the end of the incubations. The actual mean acrolein concentrations at the beginning and the end of the incubations were 5, 15, and 55 $\mu$M in the control incubation and in the incubations with the addition of 10 and 50 $\mu$M acrolein exogenously, respectively.

### TABLE 1

<table>
<thead>
<tr>
<th>Inhibition Assay Component</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, −NAD</td>
<td>8</td>
</tr>
<tr>
<td>Control, +NAD</td>
<td>0</td>
</tr>
<tr>
<td>HCY, 10 $\mu$M −NAD</td>
<td>75</td>
</tr>
<tr>
<td>HCY, 10 $\mu$M +NAD</td>
<td>78</td>
</tr>
<tr>
<td>CEPM, 20 $\mu$M</td>
<td>21</td>
</tr>
<tr>
<td>CEPM, 200 $\mu$M</td>
<td>39</td>
</tr>
<tr>
<td>PM, 60 $\mu$M</td>
<td>0</td>
</tr>
<tr>
<td>PM, 600 $\mu$M</td>
<td>4</td>
</tr>
<tr>
<td>Acrolein, 6 $\mu$M</td>
<td>55</td>
</tr>
<tr>
<td>Acrolein, 50 $\mu$M</td>
<td>90</td>
</tr>
<tr>
<td>Chloroacetaldehyde, 10 $\mu$M</td>
<td>6</td>
</tr>
<tr>
<td>Chloroacetaldehyde, 100 $\mu$M</td>
<td>19</td>
</tr>
</tbody>
</table>

The incubation conditions with HCY as the inhibitor were: human liver cytosol (pooled from three livers) was preincubated in the absence or presence of HCY (10 $\mu$M) and NAD (0.5 mM) at 37°C for 10 min, at which time the reaction was initiated with the addition of IAL (6 $\mu$M) and another aliquot of NAD (0.5 mM). Reactions were stopped after 5 min. The incubation conditions with CEPM, PM, acrolein, or chloroacetaldehyde as the inhibitor were: human liver cytosol (pooled from three livers), inhibitor, and IAL (6 $\mu$M) were preincubated at 37°C for 2 min and then the reaction was initiated with the addition of NAD (0.5 mM) and stopped after 5 min. Control refers to the incubation system with no inhibitor. The control activity in the presence of NAD was 4.74 nmol/min/mg protein. Data are mean of duplicates.

### TABLE 2

<table>
<thead>
<tr>
<th>Human Liver Cytosol</th>
<th>Exogenously added acrolein, $\mu$M</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>10</td>
<td>30.6</td>
</tr>
<tr>
<td>133</td>
<td>10</td>
<td>50.9</td>
</tr>
<tr>
<td>144</td>
<td>50</td>
<td>86.5</td>
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<tr>
<td>Mean</td>
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<td>88.7</td>
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<tr>
<td>SD</td>
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<td>80.8</td>
</tr>
<tr>
<td>% Inhibition</td>
<td></td>
<td>85.3</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>4.1</td>
</tr>
</tbody>
</table>

Human liver cytosol, HCY (20 $\mu$M), and acrolein (10, 50 $\mu$M) were preincubated at 37°C for 2 min and then the reaction was initiated with the addition of NAD (0.5 mM) and stopped after 5 min. Control incubations contained no exogenously added acrolein. Because acrolein is formed chemically from HCY during the incubations, the actual acrolein concentrations were measured at the beginning and the end of the incubations. The actual mean acrolein concentrations at the beginning and the end of the incubations were 5, 15, and 55 $\mu$M in the control incubation and in the incubations with the addition of 10 and 50 $\mu$M acrolein exogenously, respectively.
19.7% of the control activity before dialysis, and it returned to 94.4% of the control activity after dialysis.

The percentage of inhibition of ALDH1 activity in vivo by acrolein after CY administration was calculated based on the in vitro $K_i$ and $K_m$ for ALDH1, and the peak blood concentrations of HCY and acrolein in patients. Acrolein was estimated to inhibit ALDH1 activity by 85, 88, and 91% on days 1, 2, and 3 (24 h after the dose on day 2) of CY administration, respectively.

**Discussion**

The major finding of this investigation was that HCY inhibited ALDH activity in human liver cytosol incubations mainly through its degradation product acrolein. CEPM caused some inhibition, but only at very high concentrations. The other CY metabolites evaluated, PM and chloroacetaldehyde, did not show any significant inhibition of ALDH activity even at 10 times their respective maximal clinical plasma concentrations. The inhibition of erythrocyte ALDH1 activity by acrolein was best fit by a competitive inhibition model, independent of preincubation time and reversible by dialysis.

ALDH activity declined with HCY preincubation time in a first-order manner. The addition of the ALDH cofactor NAD did not increase the inhibition by HCY. These data indicated that HCY inhibited ALDH in a time-dependent manner but independent of ALDH catalytic activity. The most likely explanation was that a chemical degradation product of HCY inhibited ALDH. We therefore examined PM and acrolein as inhibitors and compared them to CEPM. We also included chloroacetaldehyde, a very minor metabolite of CY (Ren et al., 1997) because, as an aldehyde, it might inhibit ALDH. Acrolein was the major inhibitor of ALDH, although CEPM showed some inhibition at very high concentrations.

Because ALDH1 has been shown to be the major ALDH isozyme for the formation of CEPM from HCY (Dockham et al., 1992), the inhibition mechanism of ALDH1 by acrolein was investigated in ALDH1 prepared from human erythrocytes. Erythrocyte ALDH1 has been demonstrated to be structurally identical with the liver cytosolic ALDH1 and to have the same biochemical and kinetic characteristics as the latter (Helander, 1993). Our isoelectric focusing analysis confirmed that the ALDH1 prepared from human erythrocytes contained only ALDH1, not any other ALDH isoforms, in agreement with previous reports (Agarwal et al., 1983, Sugata et al., 1988).

Acrolein has been shown to be an inhibitor of rat liver mitochondrial and cytosolic ALDH (Mitchell and Petersen, 1988). It has also been shown to be an inhibitor of ALDH1 and ALDH2 purified from human liver (Ferencz-Biro and Pietruszko, 1984). However, the mechanism of inhibition has not been reported for human ALDH1. We found acrolein to be a competitive inhibitor for ALDH1 prepared from human erythrocytes.

Preincubation of human erythrocyte ALDH1 and acrolein for up to 10 min at 37°C did not increase the inhibition of ALDH1 activity, which indicated that the inhibition by acrolein was direct. Dialysis for 6 h at 4°C completely restored ALDH1 activity in ALDH1 preincubated with acrolein, demonstrating that the inhibition was reversible.

It is not practical to directly study the inhibition mechanism of acrolein on CEPM formation with HCY as substrate because acrolein is formed chemically from HCY. However, we were able to show that the formation rate of CEPM was decreased when acrolein was added exogenously to the incubations.

The inhibition of ALDH1 by acrolein in vivo in patients receiving CY was estimated with in vivo substrate and inhibitor concentrations and in vitro $K_i$ obtained with IAL as a substrate. Acrolein appears to be a potent inhibitor of ALDH1 activity at therapeutic concentrations of HCY and acrolein after administration of CY. The inhibition by acrolein was more pronounced on days 2 and 3 than on day 1 (the calculated uninhibited ALDH1 activity was 79% and 60% on days 2 and 3 compared with day 1), which was consistent with the lower elimination clearance of HCY on day 2 than on day 1 of CY administration as we have observed in a clinical study (Ren et al., 1998). Our estimate of the degree of in vivo inhibition of ALDH1 by acrolein is
based on the concentration of acrolein measured in blood. Acrolein is very reactive and readily forms conjugates with thiols. It has been shown that acrolein-thiol conjugates readily release acrolein (Alarcon, 1976, Ramu et al., 1996). Thus, our estimate of circulating acrolein concentration may be high. Nonetheless, the data strongly implicate acrolein as a pharmacokinetically significant inhibitor of ALDH1 in patients receiving high-dose CY.

In summary, we found that HCY inhibited ALDH activity through its degradation product acrolein in human liver cytosol incubations. The inhibition of ALDH1 activity by acrolein in incubations with ALDH1 prepared from human erythrocytes was competitive. The inhibition was independent of preincubation time and reversible by dialysis. Acrolein appears to be a potent inhibitor of ALDH1 activity at clinical concentrations of HCY and acrolein.

References